TITLE Studies on the Two Isoenzymes of NADH-Dependent Glutamate Synthase in Root Nodules of *Phaseolus vulgaris* L.

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Studies on the Two Isoenzymes of NADH-Dependent Glutamate Synthase in Root Nodules of *Phaseolus vulgaris* L.

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Declaration

All the work presented in this thesis is original research performed by myself under the supervision of Dr. J.V. Cullimore and has not been submitted for any degree. It was carried out in the Department of Biological Sciences, University of Warwick between July 1985 and September 1988. All sources of information have been acknowledged by means of references.

A paper (described below) written in collaboration with Dr. J.V. Cullimore has been accepted for publication and includes material presented in Chapter 3, 4 and 6.

Chen F-L and Cullimore J.V. (1988) Two isoenzymes of NADH-dependent glutamate synthase in root nodules of *Phaseolus vulgaris* L.: Purification, properties and activity changes during nodule development. Plant Physiol. (in press).

Summary

The specific activity of plant NADH-dependent glutamate synthase (NADH-GOGAT) in root nodules of *Phaseolus vulgaris* L., is over three-fold higher than the specific activity of ferredoxin-dependent GOGAT. NADH-GOGAT is composed of two distinct isoenzymes which can be separated from crude nodule extracts by ion-exchange chromatography.

Both NADH-GOGAT isoenzymes have been purified to apparent homogeneity and an anti-NADH-GOGAT antibody has been raised against purified NADH-GOGAT eluted from a denaturing polyacrylamide gel. They both have been shown to be monomeric proteins with similar M_rs of about 200,000 and are both specific for NADH as reductant. An investigation of their kinetic characteristics has shown slight differences in their K_ms for L-glutamine, 2-oxoglutarate and NADH, and they have different pH optima, with NADH-GOGAT I exhibiting a broad pH optimum of 8.0 whereas NADH-GOGAT II has a much narrower pH optimum of 8.5.

Subcellular localization studies have shown that both isoenzymes are located in plastids but in different cell types: NADH-GOGAT I is mainly present in the plastids of outercortex cells whilst NADH-GOGAT II is found to be located in the plastids of inner central cells of root nodules of *P.vulgaris*. NADH-GOGAT II has been found to be only present in root nodules and not other organs of *P.vulgaris*. During nodulation both isoenzymes increase in activity but the major increase is due to NADH-GOGAT II which increases over a time course similar to the increase in nitrogenase activity. The results suggest that the two isoenzymes of NADH-GOGAT play different physiological roles in nodule cells: NADH-GOGAT II is mainly responsible for assimilating ammonium produced during dinitrogen fixation whilst NADH-GOGAT I could assimilate ammonium from other metabolic pathways.

In nodules grown under Ar : O_2 and nodules formed with *Rhizobium* Fix$^-$ mutant CE108, both NADH-GOGAT isoenzymes, especially NADH-GOGAT II, have been observed to be expressed at early stages of nodulation but the specific activities of the two isoenzymes are low and remain constant at later stages of nodulation. However, exogenous NH_4^+ supplied to nodulated root systems has no effect on increasing the activities of the two NADH-GOGATs but can induce the senescence of the nodules. It could be proposed that the initial expression of NADH-GOGAT is independent of nitrogen fixation but the level of the two NADH-GOGAT isoenzymes, especially NADH-GOGAT II is affected by nitrogen fixation.

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Abbreviations

AAT	Aspartate amino transferase
AS	Asparagine synthetase
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
BPB	Bromophenol blue
BSA	Bovine serum albumin
cpm	Counts per minute
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid, sodium salt
FAD	Flavine adenine dinucleotide
Fd	Ferredoxin
Fd-GOGAT	Ferredoxin-dependent glutamate synthase
FMN	Flavine mononucleotide
GSH	Glutathione (reduced form)
Glu	Glutamate
Gln	Glutamine
Hepes	N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid
Lb	Leghaemoglobin
ME	β-Mercaptoethanol
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)

Chapter 1 Introduction

1.1. Benefits of nitrogen fixation to agriculture

In many of the worlds soils and climatic conditions nitrogen is the major nutrient limiting the growth of plants. Production of high-quality, protein-rich food is extremely dependent on availability of sufficient nitrogen. Although nitrogen is the most abundant element on the earth, only a few prokaryotic organisms can convert this very stable gas into a biological useful form (Sprent, 1979).

The importance of agriculture to man has caused many people not only scientists, to focus their attention on the application of complex nitrogen to the plants. The production of most of the worlds' main food crops, for example, wheat, rice, maize, barley and potatoes depends on the addition of nitrogenous fertilizers including natural organic forms and synthesized chemical forms. The former is the best nutrient to the plant growth, but the sources are limited and difficult to apply. Chemical fertilizers have played an important role in agriculture over the past 50 years. However, they have a number of defects at present: nitrogenous fertilizers are today mostly made from atmospheric dinitrogen by a reaction

$$N_2 + 3H_2 \rightarrow 2NH_3$$

which is known as the Haber process, performed at high temperature and

high pressure with the aid of catalyst. Approximately, 16kl of natural gas is consumed in the synthesis of 1kg of fertilizer nitrogen (Postgate, 1987). In addition to the cost of synthesis, the fertilizer production needs a sophisticated industry which also involves transport and packing costs. Chemical fertilizers only have a short term effect compared to natural fertilizers. They are readily leached from the soil, which is not only wasteful but also a source of environmental pollution, particularly leached fertilizer contaminates fresh water supplies. On the contrary, nitrogen fixation by some microorganisms does not need industrial production, packing and transport (Postgate, 1987) and biological nitrogen fixation is the major source of renewable combined nitrogen available to the biosphere. Crops fixing nitrogen by means of endosymbiotic association are a major world source of protein and soil nitrogen. For example, legumes may obtain all their required nitrogen from fixation, and may lead to an enrichment in soil nitrogen. Facing the ever-expanding world population, it is necessary to focus our attention on how to increase the productivity of the world crops especially in the developing countries. These countries tend to have much higher rates of population increase but lower increase in food production and they cannot afford to buy nitrogenous fertilizers. One way of solving these problems is to develop biological nitrogen fixation. These economic, sociological and environmental considerations have led to an increased interest in nitrogen fixation research in recent years and particularly on interactions between nitrogen fixing bacteria and host plants.

1.2. Nitrogen fixing symbioses.

(1) Symbiosis

Only a few prokaryotes have the ability to fix nitrogen. There are two kinds of nitrogen fixing organisms: bacteria and cyanobacteria. Some of these, like the bacteria *Klebsiella pneumoniae* and *Azotobacter vinelandii* and the cyanobacterium *Anabaena cylindrica* are capable of fixing nitrogen in the free living state. Others only fix nitrogen with certain plants; although some of these symbiotic prokaryotes can be induced to fix nitrogen outside the plant, they do not appear to do so in natural conditions.

Generally, host plants for symbiotic nitrogen fixing bacteria tend to be advanced plants, whereas cyanobacteria form symbioses mainly with primitive ones. Nitrogen fixing plant-microbe associations vary in complexity. The most simple one seems to be colonization of the rhizosphere of certain cereals (e.g. corn and sorghum) and grasses by the soil bacterium *Azospirillum*. In this case the two partners live side by side without the formation of specific structures. However, an exchange of metabolites is thought to take place (Okon, 1984). In the case of the symbiosis between the waterfern *Azolla* and the cyanobacterium *Anabaena azollae* the plant develops cavities in the dorsal lobes of its leaves in which the cyanobacteria live and reproduce but the association remains extracelluar. The most complex symbiotic association, involves actinomycetes (Frankia) or members of the

Rhizobiaceae. In these symbioses the microbes penetrate the plant and induce root nodules where nitrogen is fixed. Frankia forms symbioses with a number of plants,which are non-legume angiosperms, for example, *Alnus* and *Casuarina* species, whereas *Rhizobium* forms symbiosis with legume plants, with one known exception: *Parasponium*, which is the only non-leguminous genus known to be colonized by rhizobia.

Among all the symbioses, the legume-*Rhizobium* symbiosis is the most well understood and important, and accounts for the majority of biologically fixed nitrogen available for agriculture. Infection of legume roots with *Rhizobium* species results in the develpment of a root nodule structure in which the bacteria form an intracellular symbiosis with the plant leading to the biological fixation of atmospheric nitrogen. The symbiosis depends upon exchange of carbon and nitrogen between the host plant and *Rhizobium*. During this association they are beneficial to each other. The legume can supply carbon and energy sources for rhizobia survival and also a suitable environment to enable rhizobia to grow and divide. Rhizobia, in turn, provide fixed nitrogen in the form of ammonia for the legume. This ammonia is then assimilated into organic forms by the functions of a series of ammonia assimilatory enzymes and will be utilized for the plant growth. This mutually beneficial relationship between legumes and rhizobium species has been known for a long time and has been already applied to agriculture in crop rotations to raise soil nitrogen content for cereal crops growth. There is, therefore, a great scientific and agronomic

interest in understanding the interaction between these two organisms in their symbiosis in order to improve crop production.

(2) *Rhizobium*

This is a genus of gram negative soil bacteria of the family *Rhizobiaceae*, which can infect and nodulate legume roots, forming a nitrogen fixing symbiosis. Originally, *Rhizobium* was divided into 6 species based on the specificity between rhizobia and legumes. Specificity is an important characteristic of legume-*Rhizobium* associations and *Rhizobium* strains that share the capacity to nodulate a given legume or a group of legumes often are classified as a species. This is illustrated in the following table:

Table 1.1. Inoculation groups of *Rhizobium* species

Legume	*R. trifolii*	*R. leguminosarum*	*R. phaseoli*	*R. meliloti*	*R. japonicum*	*R. lupini*
Trifolium repens (White Clover)	+					
Pisum sativum (Garden pea)		+				
Phaseolus vulgaris (Bean)			+			
Medicago (Alfalfa)				+		
Glycine max (Soybean)					+	
Lupinus spp (Lupin)						+

The first four of these *Rhizobium* species are also classified as fast growers since the mean generation time of these organisms, grown in rich medium at 30° C under aerobic conditions is 3–4 h. The last two species are classified, by comparision, as slow growers with a mean generation time of 6–8 h. under similar conditions (Vincent, 1980). Recently the *Rhizobiaceae* was reclassified as *Rhizobium* for those fast growers, *Bradyrhizobium* for those slow growers (see also Table II).

The molecular genetics of rhizobia has been studied in some details over the past 15 years. The specificity for host range, based on the studies at genetic level, is now considered to be determined in *Rhizobium* species by genes on a large megaplasmid called the symbiotic plasmid (p*Sym*) which contains nitrogen fixation (*nif*), nodulation (*nod*) and host range (*hsn*) genes. The sizes of *sym* plasmids range from 150 to 1000 kilobases (kb). If the p*Sym* is transferred from one rhizobia species to another, the latter will acquire the ability to nodulate the legume species belonging to the former. *Bradyrhizobium* species do not contain symbiotic plasmids; genes involved in nitrogen fixation, nodulation and host specificity are located in the chromosome instead. The *Rhizobium* genus appears to be more closely related to *Agrobacterium* (another genus of the family Rhizobiaceae) which causes crown gall and hairy root diseases (Sprent, 1986) than the *Bradyrhizobium* genus.

Table 1.2. Some properties of the genera *Rhizobium* and *Bradyrhizobium* (Sprent, 1986)

	Rhizobium	*Bradyrhizobium*
Growth rate on standard media	fast	slow
Host plant specificity	restricted	promiscuous
Fixation of N_2 ex planta	difficult to achieve	easy
Genetic manipulation	easy	more difficult
DNA homology with *Agrobacterium*	high	lower

(3) Legume

Legumes are normally classified as the family Leguminosae which are found in both temperate and tropical areas though they are thought to be of tropical origin. They range in form from weed-like plants (e.g.clover) through flowering plants such as lupins, or bushes such as gorse, to trees (e.g. *Acacia*). Over 1200 species of legumes are recorded, of which about 10% have been examined for nodulation. About 50 have been exploited in agriculture but only 6-7 are regularly used in this way. The leguminosae is subdivided into three major sub-families, the Papilionaceae, the Mimosaceae and the Caesalpinaceae as shown in Table 1.3.

Table 1.3. Selected list of leguminosae

Species		Nitrogen Transport Compounds		Nodule Structure	
1)Papilionaceae		Asparagine	Ureide	Determinate	Indeterminate
Name	Common Name				
Pisum sativum	Garden pea	+			+
Vicia faba	Broad bean	+			+
Phaseolus vulgaris	French or kidney bean		+	+	
Trifolium repens	White clover	+			+
Medicago sativa	Lucerne	+			+
Ulex	Gorse				
Lupinus polyphyllus	Garden lupin	+			+
Lotus corniculatus	Birds foot trefoil	+			+
Melilotus officinalis	Melilot	+			+
Glycine max	Soybean		+	+	
Arachis hypogaea	Ground nut (peanut)				
Cicer arietinum	Chick-pea				
Vigna unguiculata	Cowpea		+	+	
2)Mimosaceae					
Name	Common Name				
Acacia	Includes the 'wattles'				
Xilia dolabriformis	Ironwood of India				
Mimosa pudica	Sensitive mimosa				
3)Caesalpinaceae					
Name	Common Name				
Cassis fistula	Senna				
Tamarindus indica	Tamarind				
Cercis siliquastrum	Judas tree				

The nitrogen-fixing legumes can also be classified into two groups on the basis of the major nitrogenous compounds transported from the nodules to the shoot. Pea (*Pisum sativum*), Broad bean (*Vicia faba*), and Lupin (*Lupinus polyphyllus*) etc. belong to a group that transports fixed nitrogen mainly as asparagine (Reynolds, *et al.* 1982, Christensen and Jochimsen, 1983). Soybean (*Glycine max*) French bean (*Phaseolus vulgaris*) and cowpea (*Vigna unguiculata*), on the other hand, belong to a group in which the ureides (allantoin and allantoic acid) are major products of nitrogen assimilation in the nodules (Schubert and Boland, 1984)

Legume root nodules can also be divided into two broad types; determinate and indeterminate, based on whether or not meristematic tissue persists in the mature nodule. Determinate nodules such as those of *Phaseolus vulgaris* and *Glycine max* have a meristem which ceases to divide at maturity and a continuous vascular system surrounding the nodule. Indeterminate nodules, such as those of *Pisum* and *Trifolium*, have a persistent meristem at the nodule apex and the vascular system is open in this area.

1.3. The nodule development process

The root nodule is a product of the interaction between the expression of both rhizobia and legume genomes. Nodule development is an elegant sequential process in which the two partners interact in a complex way, resulting in intricate structural and biochemical changes in each partner and the establishment of nitrogen fixation.

The nodulation process was elegantly described by Fahraeus (1957) in an enclosed slide culture of small-seeded legumes inoculated with rhizobia. A large number of steps are involved in the formation of a functional nodule, starting with recognition and infection of the host root and finishing with the development of bacteroids and the synthesis of nitrogenase and other essential proteins. These processes are described in six steps as follows (1) attraction of rhizobia to the roots of legume, (2) deformation of root hair and the formation of infection thread, (3) initiation of nodule meristem, (4) growth of rhizobia in infection thread and intracellular "release" of bacteria from the infection thread to host cytoplasm, (5) the differentiation of bacteroids, (6) the establishment of symbiotic association and the induction of nitrogenase and other nodule specific proteins.

The first obvious symptom when the rhizobia are inoculating a legume is the deformation of root hairs and the clumping of the rhizobia at the tip of the root hair cells. The recognition between rhizobia and legume root hair is specific. Hamblin and Kent (1973) proposed that this specific recognition involved a binding of the plant lectins to unique

carbohydrates from the rhizobia. A year later, Bohlool and Schmidt (1974) reported that soybean-nodulating rhizobia interact specifically with soybean lectin from seeds and this phenomenon was also observed in several other legume-*Rhizobium* symbioses (Dazzo and Gardiol, 1984) and this led to the formulation of "the lectin recognition hypothesis." After specific recognition of each other, very marked curling of the root hair tip is induced. Then some rhizobia entrapped within the tight curl penetrate the root hair cell wall and induce the formation of a tubular structure called the infection thread which is of host origin. The formation of infection thread is a sign of "successful" infection. The growth of infection thread follows the movement of the nucleus to the root hair base, where it then penetrates and enters the underlying root cortex. During the growth of the infection thread through three to six layers of root outer cortical cells, meristematic activity is initiated in a small group of cells in the inner root cortex directly in front of the tip of the infection thread. The growth of thread continues into these meristematic cells and the bacteria are then "budded off " and released into the host cell cytoplasm. The rhizobia are still enclosed by the infection thread membrane which becomes the peribacteroid membrane. The bacteria then divide, but all the time the plant origin peribacteroid membrane is surrounding them, failure of this results in senescence and death of the infected cell. All infected root nodule cells show a high degree of cellular organization, they contain plastids, mitochondria, endoplasmic reticulum, Golgi bodies, vacuoles and small cytoplasmic vesicles etc. despite large numbers of bacteroids

being present in these cells. In legume root nodules the central tissue enclosed by the cortex consists not only of cells that become greatly enlarged and heavily infected with rhizobia but also of many smaller uninfected cells interspersed among the infected ones (Bergersen and Goodchild, 1973). Studies on soybean nodules showed that an ultrastructural differentiation takes place in the uninfected cells which is distinctly different from changes in the infected cells.The principal changes are a marked enlargement of the microbodies as a proliferation of smooth endoplasmic reticulum (SER). It has been suggested that the uninfected cell structure is related to the participation of these cells in metabolic transport of compounds arising from recently fixed nitrogen and that they may also function in gaseous diffusion.

Variations occur among different legumes in the structure of nodules depending upon whether meristematic activity continues around the periphery of the original meristematic zone of the nodule rather than just at the apex. By their morphology, two main categories of leguminous nodules can be recognized: determinate and indeterminate nodules (Corby *et al*., 1983). Tropical legumes, such as French bean and soybean develop determinate nodules, which are round in their shapes since meristematic cells in these nodules do not divide any more at maturity. Increase in nodule size following the initial phase of infection of the meristem, appears to result largely from an increase in the volume of infected cells. Instead temperate legumes such as pea and clover develop the latter. Nodules are elongated, the meristematic cells

continuously divide and become infected as growth of the nodule proceeds. Nodule morphology is the result of a developmental program under control of the host plant, because for a number of *Rhizobium* strains it has been demonstrated that one and the same *Rhizobium* strain can induce determinate nodules on one host, indeterminate nodules on the other (Dart, 1977).

Five different zones can be defined in indeterminate nodules: (1) a zone of white meristematic cells near the nodule apex, (2) a zone of infection or thread invasion, (3) an early symbiotic zone in which the bacteriods are dividing rapidly within the plant cells which themselves contain considerable amounts of starch, (4) a late symbiotic zone in which the plant cells are packed with N_2-fixing bacteriods and which are a deep pink colour due to the presence of leghemoglobin in infected cells, (5) a zone of senescent tissue which is greenish-brown because of the degradation products of heme. In spherical nodules the older tissue tends to occur toward the centre of the nodule. Demarcation of the zones in this type of nodule is not as definite as in elongated nodules and the onset of cell senescence in spherical nodules may occur almost simultaneously over a major portion of the infected tissue.

1.4. Nitrogen fixation.

Once the bacteroids have differentiated, the nitrogen fixation starts. The principle change involves the expression of nitrogenase (E.C.1.18.2.1.). This often occurs between day 10 and 12 after inoculation and then increases rapidly during nodule development.

The importance of nitrogenase in symbiotic association, has led to a lot of interest in studying the structure and properties of this enzyme.

In 1960, a group of workers in the Central Research Laboratory of Du Pont de Nemours (a large chemical firm in U.S.A.) obtained the first active preparation of nitrogenase from *Clostridium pasteurianum* which could convert nitrogen into ammonia, a reaction proved by using the isotope of $^{15}N_2$ and isolating $^{15}NH_3$. Nitrogenase was subsequently purified from bacteroids of soybean nodules and has now been extracted from over 30 microbes.

The ability to fix nitrogen by nitrogenase is usually expressed in terms of the amount of acetylene reduction which is based on the observation that nitrogenase not only reduces N_2 but also some other triply bonded molecules into double bonded ones, such as HC≡CH (acetylene) HC≡N (hydrogen cyanide), HN_2≡N (hydrogen azide), N≡NO (nitrous oxide) etc. Among these chemicals, acetylene is especially important because the ethylene produced by nitrogenase activity can be detected rapidly and sensitively by gas chromatography.

This reaction, therefore, is routinely used for measuring nitrogen fixation.

Nitrogenase is one of the most complicated systems in present-day enzymology. It actually consists of two distinct components; the large one usually called Mo-Fe protein (component I) binds the reducible substrate. The smaller one, Fe-protein (component II) transfers electrons to the Mo-Fe protein. Both the two components are irreversibly inactivated by oxygen. Nitrogen fixation is not performed at all in the presence of high partial pressures of O_2. The Mo-Fe protein contains two atoms of Mo and 24 to 32 atoms of Fe and S per molecule. There appear to be four [4Fe-4S] clusters and two [$MoFe_6S_8$] clusters. The two [$MoFe_6S_8$] clusters comprise the MoFe cofactor (Vance *et al.*, 1988). Evidence shows that this is the site at which dinitrogen is reduced to ammonia. The Fe-protein contains two apparently identical sub-units together with a cluster of 4 iron and 4 labile sulphur atoms. The two components join together and function coordinately to perform the nitrogen fixation. Nitrogenase activity also requires ATP, combined with Mg^{2+}, as energy source. Reduction of nitrogen to ammonia requires 6 electrons, and 12 ATP molecules, according to the reaction equation.

$$N_2 + 12\,ATP + 6e^- + 6H^+ \longrightarrow 2NH_3 + 12ADP + 12Pi$$

Yet the energy requirement for nitrogenase activity is much greater than that shown in the reaction equation. The reason for this was

finally found in the early 1980s', nitrogenase evolves H_2 as a by product of the nitrogen fixation reaction.

$$2H^+ + 2e^- + 4ATP \longrightarrow H_2 + 4ADP + 4Pi$$

Nitrogen fixation appears to be more accurately represented as an eight-electrons process reducing N_2 to $2NH_3$ plus H_2 as follows:

$$N_2 + 8H^+ + 8e^- + 16ATP \longrightarrow 2NH_3 + H_2 + 16ADP + 16Pi$$

All nitrogenase preparations studied so far have been found to catalyze this reductant and ATP dependent H^+ reduction to H_2 during the reduction of N_2 (Dixon *et al.*, 1981, Schubert and Evans, 1976, Shanmugam *et al.*, 1978). The evolution of hydrogen during nitrogen fixation is believed to be an inherent property of the nitrogenase reaction and to be an energy draining process (Schubert and Evans, 1976). Hydrogen evolved by nitrogenase is also a competitive inhibitor of nitrogen fixation, and Dixon *et al.* (1981) have suggested that hydrogen could accumulate to inhibitory level (c. 10%, v/v) within the nodule. However some nitrogen fixing organisms, including some rhizobium species, contain an oxygen-dependent enzyme system called "uptake hydrogenase", which can recycle the H_2 released and the electrons at the same time during nitrogen fixation. This system was originally thought to be beneficial to the plant since the ability of

nitrogen fixation is limited by the supply of energy from oxidative phosphorylation, which is ultimately derived from the plants carbon compounds and any process that reduces the energy cost for nitrogen fixation might be expected to result in increased nitrogen fixation and consequently in increased plant growth. The loss of more than 25% of the energy flux through nitrogenase as hydrogen evolution might be compensated, at least in part, by the activity of the hydrogen recycling system. This uptake hydrogenase reduces the concentration of free O_2 during the reduction of H_2. Thus this hydrogen recycling system can also play a role in protecting nitrogenase from oxygen. However, carefully controlled experiments with Hup^- mutants showed that there were no differences in nodule nitrogen fixation efficiency between near-isogenic Hup^+ and Hup^- strains (Cunningham *et al.*, 1985). The significance of the presence of the Hup gene has therefore become difficult to explain.

Besides Fe-Mo nitrogenase, recently Robson *et al.* (1986) reported that there existed an alternative vanadium containing nitrogenase from *Azotobacter chroococcum*, under conditions of Mo deficiency, which does not require the structural genes for conventional nitrogenase.

1.5. Ammonia assimilation

(1) Glutamate Synthase Cycle

In root nodules, the first product of nitrogen fixation ammonia is excreted from the bacteroids and then assimilated by the plant. Once formed, ammonia could be assimilated by various pathways. For many years the assimilation of ammonia was thought to be only via the reductive amination of 2-oxoglutarate to give glutamate as catalyzed by glutamate dehydrogenase (GDH, EC 1.4.1.3.).

$$\text{2-oxoglutarate} + NH_3 + \text{NAD(P)H} + H^+ \xrightarrow{\text{GDH}} \text{glutamate} + \text{NAD(P)}^+ + H_2O$$

GDH activity has been found in a number of plant species and is abundant in most tissues. Initial ^{15}N studies of Kennedy (1966) on Serradella nodules suggested that the ammonia from nitrogen assimilation was incorporated directly into glutamate. However its high K_m for NH_3 and its virtual disappearance in *Klebsiella aerogenes* under conditions of low N availability when NH_3 assimilation was still occurring, resulted in the discovery of an alternative route of ammonia assimilation in this organism in 1970, combining glutamine synthetase (GS) and a newly discovered enzyme glutamate synthase(GOGAT EC2.6.1.53.)(Tempest *et*

*al.*1970). Miflin and Lea(1976) suggested that assimilation of ammonium occurs in the plant cytosol by the GS/GOGAT pathway, rather than by GDH, following a reanalysis of Kennedy's data from $^{15}N_2$ labelling of Serradella nodules. Morever Miflin and Lea supposed that GS/GOGAT pathway is the major, perhaps only route of ammonia assimilation in higher plants (1976). In this pathway GS is responsible for the initial assimilation of ammonia into glutamine and GOGAT catalyzes the transamidation of glutamine amido-nitrogen to the α-amino position of 2-oxoglutarate to form two moles of glutamate. Because the two enzymes are dependent on each other for the provision of substrate, their activities constitute a cycle which has been termed the glutamate synthase cycle (Miflin and Lea 1980).

$$\text{Glutamate} + \text{ATP} + NH_3 \xrightarrow{\text{(GS)}} \text{Glutamine} + \text{ADP} + \text{Pi} + H_2O$$

$$\text{2-oxoglutarate} + \text{Glutamine} + \text{NADH (or NADPH or Fd red.)} + H^+ \xrightarrow{\text{(GOGAT)}} 2\ \text{Glutamate} + NAD^+ \text{ (} NADP^+ \text{ or Fd ox.)}$$

More elegant studies by Meeks *et al.*(1978) on soybean nodules using $^{13}N_2$, a short-lived radioactive isotope of N, demonstrated that NH_4^+ is first incorporated into the amide position of glutamine in the reaction catalyzed by GS. This incorporation is blocked by methionine sulfoximine, an inhibitor of GS (Meeks *et al.*, 1978). The amide group is subsequently transferred to the 2-carbon of oxoglutarate in the reductive amination

reaction carried out by GOGAT, which is blocked by azaserine, a general inhibitor of amide-group transfer reaction (Meeks *et al.* 1978). Similar results have been obtained by Ohyama and Kumazawa (1980). Because GS has a much lower K_m for ammonia than GDH, the glutamate synthase cycle can function and maintain very low concentrations of ammonia in the tissue and would be important to maintain a gradient favouring continued NH_4^+ export by bacteroids (Rawsthorne *et al.*, 1980). Further evidence for the GS/GOGAT pathway as the primary ammonia assimilatory route comes from the developmental studies of nodule enzyme activities. Robertson *et al.* (1975a,b.) reported that both GS and an NADH-dependent GOGAT are induced during lupin nodule development in parallel with nitrogenase activity and leghemoglobin content and in alfalfa both GS and GOGAT decline along with nitrogenase activity when nodulating roots are deprived of their shoot system. These enzymes increase in activity again as nitrogenase activity increases during nodule rebuilding (Groat and Vance, 1981), yet changes in the activity of GDH do not appear to be correlated with nitrogen fixation. During nodule development in soybeans GDH activity declined markedly at a time when nitrogen fixation was increasing (Sen and Schulman 1980).

In view of all the above evidence, it is convincing that in legume root nodules ammonia excreted by the bacteroids is assimilated only via the glutamate synthase cycle.

Work on other parts of the plant has already shown that the glutamate synthase cycle is the major route of ammonia assimilation in higher plants. Ammonia in higher plants is derived both from the plant's

primary nitrogen sources (ammonia, nitrate or , for legumes, dinitrogen) and from a number of internal nitrogen-cycling pathways such as photorespiration, amino acid catabolism and phenyl propanoid metabolism. The glutamate synthase cycle is therefore essential not only for primary nitrogen assimilation but also to maintain the general nitrogen economy of the plant.

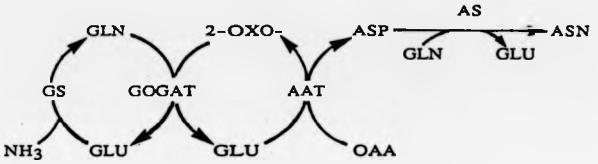
(2) Asparagine and ureide transport pathways:

Nitrogen-fixing plants can be classified as amide transporting plants and ureide transporting plants based upon the composition of the xylem fluid collected from excised nodules or nodulated root systems. Lupins, peas, the "temperate legumes", belong to the first group, soybeans, cowpeas and beans, mainly the "tropical legumes", belong to the second group.

(a) Asparagine transport pathway

The predominant nitrogen compounds in the xylem of lupins and other amide.transport legumes is asparagine. Using $^{15}N_2$ labelling of Serradella nodules the labelling pattern suggested that asparagine was an end-product of the nodule nitrogen assimilatory pathway following primary incorporation into glutamine and glutamate. In work on soybean nodules, label from $^{15}NH_4^+$ was initially detected in glutamine-amido N

followed by glutamate and asparagine-amido N (Fujihara and Yamaguchi, 1980). In these experiments aspartate was also rapidly labelled but only after glutamate, and it was obvious that this compound was an intermediate in the nitrogen metabolic pathway (see Schubert review 1986) In *Vicia* labelling studies using $^{14}CO_2$ have also identified glutamate and aspartate as primary products of assimilation, with label later appearing in asparagine (see Schubert review 1986). Robertson *et al.* suggested therefore that asparagine was probably synthesised as follows: (1980)



From this route it is obvious that another two enzymes besides GS and GOGAT are involved in asparagine synthesis. These are aspartate aminotransferase (AAT EC2.6.1.1.), and a glutamine-dependent asparagine synthetase (AS EC 6.3.5.4.). In lupin, the activities of AAT and AS both increased considerably during nodule development. No asparagine synthetase activity could be detected in either the bacteroid fraction of the nodules or in cell-free extracts of the free-living rhizobia grown in a broth

or minimal medium. Above results suggested that plant enzymes are involved in the assimilation of the ammonia produced from the nitrogen reduction in the bacteroid.

(b) Ureide synthesis

In tropical legumes like soybean, bean and cowpea, ureides (allantoin and allantoic acid) represent the major products of nitrogen assimilation in nodules. Synthesis of these componds is directly associated with the assimilating process of symbiotically fixed nitrogen in nodules (Fujihara and Yamaguchi 1980)

^{15}N-labelling studies in soybeans and other ureide exporting legumes showed that recently fixed nitrogen was incorporated into allantoin and allantoic acid within the nodule and then exported to other parts of the plant (Matsumoto *et al.*, 1977a, 1977b, 1978). Studies on activities of nitrogen assimilatory enzymes showed that during the early stages of nodule development both aspartate aminotransferase and asparagine synthetase also increased in soybeans but between 17 and 19 days after inoculation of soybean nodules, the activity of asparagine synthetase declined greatly and the concentration of asparagine was very low. All these show that asparagine synthetase does not provide the major route for ammonia assimilation in the nodules of soybeans. Two possible routes for ureide biosynthesis have been proposed by Reinbothe and Mothes (1962),

The first one involves the condensation of urea and a two-carbon compound such as glyoxylate, the second involves the oxidative catabolism of purines. Although in 1953 Mothes suggested that the latter pathway was unlikely, evidence supporting the condensation pathway has been limited to labelling studies in certain fungi and in banana. Several lines of evidence suggest that the second route i.e. the purine catabolic route to ureide biosynthesis is present. Firstly, the presence of xanthine dehydrogenase (XDH), uricase and allantoinase, enzymes of purine catabolism are at high levels in nodules of the ureide-exporting plants cowpea, soybean and garden bean (Schubert review, 1986), but at low level in the asparagine transporting legumes such as lupin (Reynolds *et al.*, 1982) and pea (Christensen, Jochimsen 1983).Secondly,the levels of these enzymes increase in response to the onset of nitrogen fixation (Atkins *et al.*, 1980, 1981,1984, Reynolds *et al.*, 1979,1982). Thirdly, the addition of allopurinol, an irreversible inhibitor of XDH, results in a decrease in levels of ureides in nodules and ureide export in the xylem and an increase in xanthine in the nodule (Atkins *et al.*, 1980, Fujihara and Yamaguchi, 1978, 1980). The pathway for ureide synthesis is shown as follows:

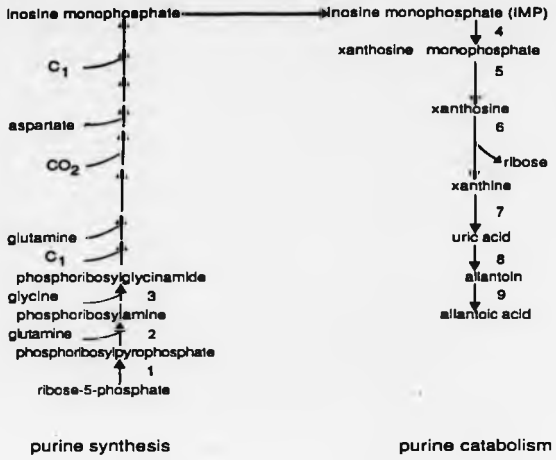


Fig. 1.1. A proposed pathway for ureide biosynthesis. The enzymes are (1) Phosphoribosylpyrophosphate synthetase, (2) Phosphoribosylamidotransferase, (3) Phosphoribosylglycinamide synthetase, (4) Inosine monophosphate dehydrogenase, (5) 5' nucleotidase, (6) Purine nucleosidase, (7) Xanthine dehydrogenase, (8) Uricase, (9) Allantoinase

De novo purine synthesis requires the precursors, ribose, glutamine, glycine, aspartate and methylene-tetrahydrofolate and the end product is inosine monophosphate (IMP). Then IMP is oxidized and degraded to form allantoin and allantoic acid. Ureide formation is carried out by a series of reactions involving the enzymes shown in Fig 1.1, not only those directly involved in purine catabolism but also those responsible for purine and purine precursor biosynthesis. Levels of all these enzymes are increased several fold in nodules of ureide-producing legumes over those found in nodules of amide-producing plants (Atkins, 1984, Reynolds *et al.* , 1982, Schubert and Coker, 1982). Boland and Schubert (1982) confirmed that purines were synthesized *de novo* and oxidized *in vivo* by showing that $^{14}CO_2$ is incorporated into xanthine. This ^{14}C should be incorporated into the purine ring at the C6 position, but it should not be detected in ureides due to the loss of this carbon during purine oxidation. By blocking the complete oxidation of the purine with allopurinol, Boland and Schubert (1982) showed that xanthine labelled specifically at the C6 position. Even without the inhibitor, ^{14}C xanthine was also detectable rather than IMP, the end product of purine synthesis. These results further show that purine synthesized *de novo* are rapidly oxidized in nodules as a part of ureide biogenesis. The synthesis of xanthine from IMP was also confirmed using $^{14}CO_2$ by Boland and Schubert (1982).

1.6. The regulation of plant gene expression in nodules

The development of an effective nitrogen-fixing symbiosis involves complex interaction between the host plant and rhizobium symbiont. An effective symbiosis is accomplished by differentiation of bacteria into bacteroids on the one hand and differentiation of plant cell into a root nodules on the other. Both legume and rhizobial genes are involved during the symbiotic association. Over the last few years interest in the role of the plant in the symbiosis has considerably increased. Genetic and biochemical evidence indicates that the host plant plays an important role in the nodulation process. A group of proteins encoded by the plant genome called nodulins (Legocki and Verma, 1980) are induced specifically. They appear to play an important role in establishing and maintaining the symbiotic association. Nodulin genes are differentially expressed during nodule development. Some genes are expressed in the stage of nodule development during which the nodule structure is formed. They are called early nodulin genes. The majority of nodulin genes are expressed around the onset of nitrogen fixation. These genes are called late nodulin genes which are important in nitrogen fixation.

Over 30 different nodulins have been detected from several legumes. Studies with soybean, pea, french bean and alfalfa showed that nodulins were only found in root nodules and not in roots or other plant organs (Nap 1988, Vance *et al.*, 1985). Nodulins are important in symbiotic association because they may have specific functions in the formation of

nodule tissue after de-differentiation and proliferation of cortical cells, in the transport of substrates to the bacteriods or assimilated ammonia to other parts of the plant. Based upon their possible functions, nodulins have been divided into three categories (Fuller *et al*., 1983):

(1) proteins that support bacteroid functions and thus facilitate nitrogen fixation.

(2) enzymes involved in specific nitrogen assimilation and carbon metabolism of nodule.

(3) proteins responsible for the maintenance of nodule structure.

Although a lot of work has been done on gene expression and regulation of some nodulins, the functions of most nodulins remain unknown. However the functions of at least one or more nodulins from each type have been best identified. They are: the most abundant protein leghemoglobin (Lb) which belongs to the first type of nodulins; among the second group are glutamine synthetase (GS, EC 6.3.1.2.), aspartate aminotransferase, (AAT, EC.2.6.1.1.), uricase (EC.1.7.3.3.) and xanthine dehydrogenase (XDH, EC. 1.2.1.37). (all ammonia assimilatory enzymes) and sucrose synthase (EC.2.4.1.13.) (involved in carbon metabolism.); an example of the third type of nodulins is the peribacteroid protein Nodulin24.

The most abundant plant gene product found in all legume nodules is Lb. It is the only nodulin the presence of which can be observed by the eye. Lb is a myoglobin-like protein that serves as O_2 carrier and regulates the

O_2 tension within the nodule (Whittenberg *et al.*, 1974) to protect nitrogenase from inactivation. It consists of two parts, pigment part–heme, which is produced by the bacteria (Nadler and Avissar, 1977) and apoprotein–globin which is synthesized by the plant (Baulcombe and Verma, 1978). In all legumes studied to date more than one Lb is found in the root nodule, and the Lbs are encoded by more than one gene. Work on soybean root nodules has shown that Lb consists of four major components denoted Lba, Lbc1, Lbc2 and Lbc3, and some less characterized minor components (Vance and Johnson, 1983). The soybean Lb genes are encoded in the plant genome as a small family of genes which are specifically activated in response to infection of the root with *Bradyrhizobium japonicum*. By using "Western" blotting technique, the result shows that the induction of leghemoglobin during nodule development was two or three days earlier than, but paralleled, the induction of nitrogenase activity.

A lot of work has been done on the second group of nodulins. GS is the first enzyme involved in the assimilation of ammonia excreted by the bacteroids and has been widely studied in different plant species. Biochemical studies have shown that higher plant GS is an octameric enzyme of M_r 360,000 located in both the chloroplasts and cytosol (McFarland *et al.* 1976, McNally *et al.* 1983). In *Phaseolus vulgaris*, there are four distinct forms of the holoenzymes which can be separated by ion–exchange chromatography. Two of these are found in leaves in the cytosol and chloroplast; one is found in the cytosol of both roots and nodules, the fourth one is cytosolic and nodule–specific. Biochemical studies have shown that these isoenzymes consist of three cytosolic and a

plastidic GS polypeptide which assemble to form the distinct octameric GS isoenzymes (Cullimore and Bennett 1988). Recent studies show that these different GS polypeptides are all encoded by four different nuclear genes: gln-α , gln-β , gln-γ and gln-δ . All four genes appear to be transcribed in nodules, but gln-γ gene is expressed nodule-specifically and can be considered to produce a nodulin (Cullimore and Bennett 1988). In *P. vulgaris* GS activity increases 16-fold from days 10-18 following inoculation of the plants with *Rhizobium* and this increase occurred over a time course similar to the synthesis of nitrogenase in the bacteroids and Lb in the plant cell cytosol. This increase in activity is due mainly to the expression of the gln-γ gene which produces a cytosolic GS polypeptide and isoenzyme. In nodules of lupin, soybean and pea, the same pattern of changes in GS activity has also been observed (Cullimore and Bennett 1988).

Another example for the second type of the nodulins is aspartate aminotransferase (AAT), which is present in both ureide- and amide-transporting legumes. AAT exists in the plant fraction of the nodule as two isoenzymic forms: AAT-P_1- a soluble enzyme also present constitutively in roots, and AAT-P_2- a nodule specific enzyme whose induction is positively correlated with the onset of biological nitrogen fixation. AAT activity in the plant cytosol fraction of lupin nodules increased 5-fold in the period 10 to 18 days after rhizobia inoculation (Reynolds and Farnden, 1979). Examination of the fraction by polyacrylamide gel electrophoresis revealed that the intensity of AAT-P_2 increased during nodule development, whereas the intensity of AAT-P_1 remained constant, which

suggests that the increase in AAT activity during nodule development is due to the induction of a nodule specific isoenzyme AAT-P_2.

A third example of the second type of nodulins is uricase. It is the second most abundant protein in the cytoplasm of soybean root nodules. Bergmann *et al.*(1983) reported that the previously unidentified nodulin-35 was the 33-kD subunit of a specific soybean root nodule uricase (uricase II) located in uninfected cells of nodule tissue. The ureides are the principal form in which fixed nitrogen is transported from soybean nodules. (Pate and Atkins, 1983). Uricase activity found in young uninfected soybean roots is due to another form of the enzyme (uricase I) which is of 190 kD, has maximum activity at pH 8.0 and does not contain any subunit corresponding in size to nodulin-35. They are two distinct enzymes since monospecific antibodies prepared against uricase II (nodulin-35) showed no cross reactivity. Uricase I declines at a time when nodulin-35 appears and uricaseII activity increases at a time course similar to GS and nitrogenase activity during nodule development (Bergmann , *et al.*, 1983). These results are consistent with the concept that a nodule specific ureide metabolism takes place in peroxisomes of uninfected cells, and suggest the participation of uricase II in this pathway.

Recently, another nodule specific ammonia assimilatory enzyme xanthine dehydrogenase (XDH) from soybean root nodules has been purified and studied (Nguyen *et al.* 1986). The results also shows that this enzyme activity is induced parallel with those of other ammonia assimilatory enzymes.

Some nodulins are induced to function in carbon metabolism during

nodule development. Nodule specific sucrose synthase from soybean nodules has been purified recently. It is a tetrameric enzyme composed of identical monomers with an apparent M_r of 90,000 and represents an abundant protein in the nodule cytosol (Thummler and Verma, 1987). Thummler and Verma also reported that the nodulin-100 transcript of soybean encodes sucrose synthase. This enzyme catalyzes the following reversible reaction:

$$\text{Sucrose} + \text{UDP} \rightleftharpoons \text{UDP-glucose} + \text{D-fructose}.$$

and appears to be involved in the cleavage of sucrose in soybean nodule tissue. Antibodies raised against this enzyme cross-reacted with the hybrid-released translation product of nodulin-100 cDNA, suggesting that nodulin-100 is the subunit of this enzyme.

Some of the nodulins were located within the peribacteroid membrane, such as studies on nodulin-24 recently by Katinakis and Verma (1985). *In vitro* translation and immunological techniques suggests that it is a precursor and is processed cotranslationally into a polypeptide of M_r 20,000 which is a component of the membrane envelope enclosing the bacteroids synthesized during symbiosis with *Rhizobium*.

A lot of interests have now turned to investigate the regulation and expression of nodulins. The expression of genes for nodulins appears to correlate with the induction of densely cytoplasmic host cells and to some extent, with bacterial release from infection threads (Norris *et al.*, 1988). For example , in alfalfa nodules induced by *Rhizobium exo* mutant without release of the bacteria from the infection threads, no Lb or nodule-specific

GS was expressed (Dunn *et al.*, 1988). The correlation between nodule structure and nodulin gene expression is best studied in the plant species, vetch and pea. *Rhizobium* strains mutated in one of the *nif* or *fix* genes induce the formation of nodules on pea and vetch that are morphologically similar to nodules induced by wild-type *Rhizobium*. In these nodules, rhizobia differentiate into the characteristic bacteroidal shape and all nodulin genes are expressed (Govers *et al.* 1985, 1987). Thus nitrogen fixation is apparently not essential for the expression of nodulin genes. Another evidence to support this conclusion is from experiments in which legume plants are grown under $Ar:O_2$ rather than under $N_2:O_2$. The nodules induced by wild type of *Rhizobium* are morphologically similar to nodules grown under nitrogen; and although nitrogen fixation is absent, all nodulin genes are expressed. Experiments with some cloned nodulin genes involved in ammonia assimilation have indicated that expression of these genes is not coupled to activation of the nitrogenase genes of *Rhizobium* (Fuller *et al.* 1984, Marcker *et al.* 1984) or nitrogen fixation (Govers *et al.*1985, Lang-Unnasch *et al.* 1985). For example, studies on expression of nodule specific GS during nodule development showed that the induction of Lb and GS gene take place independent of nitrogenase activity (Sengupta-Gopalan and Pitas 1986, Padilla *et al.* 1987). Therefore, it could be proposed that all enzymes involved in ammonia assimilatory might be induced in close association with the intracellular infection by *Rhizobium*. Yet it could not be ruled out the possibility of the involvement of common factors in regulating the activation of the genes of nitrogenase, leghemoglobin and ammonia assimilatory enzymes. However, it seems

premature to draw meaningful generalizations from the date available for the regulation of the expression of the nodulin genes. The expression of nodulin genes is a rather complicated process controlled at different levels and involving various factors.

1.7 Two forms of glutamate synthase in higher plants:

In higher plants, there exists two forms of glutamate synthase, one utilizing pyridine nucleotide as reductant, the other utilizing ferredoxin. NAD(P)H-dependent glutamate synthase was first identified in cultured carrot cells and pea roots (Dougall 1974, Fowler *et al.*1974). In the same year ferredoxin-dependent glutamate synthase (Fd-GOGAT) was discovered in green leaves of pea by Lea and Miflin (1974).

In higher plants both NADH-dependent glutamate synthase (NADH-GOGAT) and Fd-GOGAT have been purified and characterised. Fd-GOGAT has been shown to be most abundant in green leaves of plants and is located in the chloroplast. It has been purified from a number of higher plant species and organs (Suzuki and Gadal, 1984) and generally these reports agree that it is a monomeric protein of M_r about 140,000. However in green leaves of rice, Fd-GOGAT appears to have a M_r of 250,000 consisting of two identical polypeptides of M_r of 125,000. Work on photorespiratory mutants of *Arabidopsis thaliana* (Somerville and Ogren, 1980) and barley (Kendall *et al.* 1986) have shown that this enzyme is essential for the reassimilation of photorespiratory ammonia in leaves. The enzyme has also been detected in non-green tissues such as roots, cultured cells, etiolated leaves and in legume nodules although its precise role and the provision of its reductant in these tissues is unclear (Oaks and Hirel, 1985). Studies on the Fd-GOGAT of rice green and etiolated leaves

show that they are immunochemically identical proteins whereas Fd-GOGAT from rice root and soybean nodules are recognized by the antibodies but are antigenically different from the leaf enzyme(Suzuki and Gadal, 1984). Antibodies raised against the rice leaf Fd-GOGAT failed to cross-react with NADH-GOGAT from several organs and species, thus suggesting that Fd-GOGAT and NADH-GOGAT are two distinct enzyme proteins (Suzuki and Gadal, 1984).

NADH-GOGAT was first purified from root nodules of lupin (Boland and Benny 1977). Subsequently this enzyme was purified from soybean cell cultures (Chiu and Shargool, 1979), etiolated pea shoots (Matoh and Takahashi, 1980), and *Chlamydomonas* (Marquez *et al.*1984). It appears to be an iron-sulphur flavoprotein with a single subunit of M_r about 230,000 and is specific for NADH as reductant, showing little or no activity with NADPH or ferredoxin. In legume nodules, a lot of experiments have shown that the NADH-dependent enzyme is present mainly in the host plant cells, and is absent or low in activity in bacteroids (Boland *et al.*, 1977, 1983, Awonaike *et al.*, 1981, Shelp *et al.*, 1983). Sub-cellular fractionation studies on nodules of *Phaseolus vulgaris* (Awonaike *et al.*, 1981), soybean (Boland *et al.*, 1982), and lupin (Shelp *et al.*, 1984) and on leaf callus cultures of *Bouvardia ternifolia* (Murillo *et al.*, 1985) indicate that this enzyme is associated largely with the plastids.

As mentioned above, it appears that NADH-GOGAT and Fd-GOGAT are two different protein enzymes which differ in molecular weight and reductant specificity. The major properties of NADH-GOGAT

and Fd-GOGAT in higher plants are summarized in the following table:

Table 1.4. A Comparison of NADH-GOGAT and Fd-GOGAT of Higher Plants

	NADH-GOGAT	Fd-GOGAT
MW	200,000~230,00	~140,000 250, 000 (from green leaves of rice)
No of subunit	1	1 2 (from green leaves of rice)
Location	non-green tissues: nodules, roots etc. plastids	green leaves: chloroplast, plastids of other tissues
Reductant	NADH	ferredoxin
pH optimum	7.5~8.5	6.9~7.5
K_m NADH	4~13 μM	
Fd		2~5.5μM
GLU	400~1000μM	100~1000μM
2-OXO	39~ 960μM	7~ 70μM
Stability	very unstable room temp.	
Cofactor	FAD/FMN non-heme iron, acid-labile sulphur	ferredoxin, FAD

Although Fd-GOGAT and NADH-GOGAT both catalyze the transamidation of glutamine amido-N to the α-amino position of 2-oxoglutarate to form two moles of glutamate, the table shows clearly that the two enzymes are greatly different in structure, kinetic properties, and localization.

Developmental patterns of the two enzymes are also different. In early growth stages of higher plant seedlings, NADH-GOGAT activity is higher than Fd-GOGAT, this activity gradually decreases whereas Fd-GOGAT activity increases during seedling development. In mature green tissues Fd-GOGAT is the main form, and NADH-GOGAT activity is either absent or contributes less than 5% of the total activity in mature shoots (Stewart and Rhodes, 1978; Matoh and Takahashi, 1982). Wallsgrove *et al.* (1982) reported that during greening of etiolated shoots of pea and barley, Fd-GOGAT activity increases rapidly and in light grown leaves Fd-GOGAT activity is 30-40 fold higher than NADH-GOGAT activity. Fd-GOGAT activity has also been shown to be present in soybean root nodules where it appears to be about twice as high as NADH-GOGAT activity (Suzuki *et al.* 1984*b*). Work on GOGAT in root nodules has shown that NADH-GOGAT activity increases during nodulation of lupin (Robertson *et al.*, 1975) and soybean (Reynolds *et al.*, 1982) over a time course similar to the increase in nitrogenase in the bacteroids. All these results suggest that the two enzymes are under different regulatory mechanisms which are closely related to their functions in different

plant cells. From *A. thaliana* mutants (Somerville and Ogren, 1980) and barley mutants (Kendall *et al.* 1986), Fd-GOGAT and NADH-GOGAT appear to be coded for by different genes.

1.8. Aim of the study

In contrast to the work on Fd-GOGAT in green tissues, much less work has been done on NADH-GOGAT. This may be due to the instability of the enzyme *in vitro* and the low abundance of the enzyme present in many tissues (Suzuki and Gadal 1984). NADH-GOGAT in root nodules plays a crucial role with GS in assimilating ammonia produced during dinitrogen fixation. Therefore it is important to study the enzyme to determine the relationship between NADH-GOGAT and Fd-GOGAT and the regulation of GOGAT and GS. Moreover in comparison to the work on GS, very little is known about the coding and expression of GOGAT in higher plants: to date no clones have been obtained for either Fd-GOGAT or NADH-GOGAT. My research project is to study NADH-GOGAT of root nodules of *Phaseolus vulgaris*. The work has been divided into the following aspects:

(1) purify plant NADH-GOGAT from *Phaseolus vulgaris* root nodules.

(2) study the characteristics of NADH-GOGAT

(3) produce polyclonal antibodies to the purified enzyme

(4) study the subcellular and cellular localization of NADH-GOGAT

(5) study the changes in activities of NADH-GOGAT during nodule

development to find out the regulatory factors affecting the expression of the enzyme.

(6) construct a cDNA library from root nodule high molecular weight polyA^+ RNA and to screen for clones coding for NADH-GOGAT.

Chapter 2

Materials and Methods

2.1. Materials.

1. Plant Materials

Phaseolus vulgaris L. cv Tendergreen was generally grown in plant growth rooms in 6" pots with perlite under 18 h light/6 h dark cycles (22° to 24°C). The light intensity was 100 μmol photons m^{-2} s^{-1}. The plants were watered with Hewitt's -N nutrient solution described on page 53. Nodules were formed by inoculating 5 day-old seedlings (day 0) with *Rhizobium leguminosarum* bv *phaseoli* R3622, previously grown for two days at 30°C in yeast extract/mannitol medium. Fig 2.1. shows a 25-day old plant of *P.vulgaris*.

For mutant experiments, seeds were surface sterilized and sown in autoclaved tissue towel until germinated and then five seedlings were transferred to 1liter beakers with autoclaved perlite filled with autoclaved -N nutrient solution. The beaker was surrounded by black paper to exclude light. 5 day-old seedlings were inoculated with *Rhizobium* mutant CE108 and wild type CE3 separately as control.

For argon experiment, seeds were soaked in running tap water overnight and then sown in vermiculite until 5 cm high and 5 seedlings were transferred into twelve 40cm diameter plastic pot with gravel, which were filled with -N nutrient solution. On the second day plants were inoculated with *Rhizobium* R4962 (day0). On day5 pots were sealed with silicone

rubber sealant and a gas mixture containing 80% nitrogen and 20% oxygen (7 pots) or 80% argon and 20% oxygen (5 pots) were supplied at a flow rate of 200ml/hr. On day15 the first nodule samples were harvested from both nitrogen supplied plants and argon supplied ones (one pot of each); for the remaining argon supplied pots, two were switched off argon and transferred to nitrogen and two were given 10mM ammonium; and the remaining two were maintained on argon. For the remaining nitrogen supplied pots, two were switched off nitrogen and transferred to argon and the remaining two were still given nitrogen. Nodules from pots of each treatment were then harvested on day16 and 19.

Nodules (Fig.2.2.) were picked on about day 20 after legume inoculation. Unless otherwise stated nodules were picked, free of the root system and stored in liquid nitrogen. For the nodule development experiment shown in Fig.6.1.-6., samples harvested at day 5, 8, and 10 consisted of the top 5 cm of the nodulating root system. For purification of NADH-GOGAT, nodules were also grown in greenhouses (21° to 24°C) with natural daylight supplemented to 14h day length with artificial lighting. For localization of NADH-GOGAT in root nodule cells, the nodules were picked on day 18 after legume inoculation and placed on ice for extraction. Soybean nodules were harvested from *Glycine max* cv Prize and a Chinese variety, Peking grown in gravel in growth rooms as described above except that the daylength was 12 h. The rhizobia strain used was *Bradyrhizobium japonicum* USDA 110.

Pea and alfalfa were grown in the same way as French bean. Pea seedlings were inoculated with *Rhizobium leguminosarum* bv *leguminosarum*. Alfalfa seedlings were inoculated with *Rhizobium meliloti*.

For experiment of ammonia effect on NADH-GOGAT, beans were soaked in running water overnight and then sown in vermiculite for 5days until the first leaf grew out. The cotyledons were picked off and the seedlings were supported by wire mesh in a tray (hydroponic system) filled with -N nutrient solution and bubbled with fresh air (Fig.2.3.). On the third day 10mM ammonium sulphate solution was given. Samples of root and leaves were harvested at 10 hours and 24 hours after giving ammonium.

The etiolated leaves, stems, cotyledons, were obtained by growing beans in dark room for 2 weeks watered with -N nutrient solution.

Nitrogenase antibody was kindly supplied by Dr. T. Bisseling, Dept. of Molecular Biology, Agriculture University, Wageningen.

Uricase antibody was kindly supplied by Dr. D.P.S. Verma, McGill University, Montreal.

Seeds were obtained from Booker Seeds Company.

Fig.2.1. A plant of *P.vulgaris* (25 days old since sowing) grown in Plant Growth Room

Fig.2.2. 20 days old nodules (since inoculation of the plant) of *P.vulgaris*

Fig.2.3. The effect of NH_4^+ on uninoculated roots of *P.vulgaris* carried out in Hydroponic system

2.2. Chemicals

All chemicals of the highest grade commercially available were obtained from :

Boehringer Corporation (London) Ltd.

BDH Chemicals Ltd.

Bio-Rad Laboratories Ltd.,

Fisons Chemicals Ltd.

Pharmacia (GB) Ltd.

Sigma Chemical Co. Ltd. (London)

Grand Island Biological Co. Ltd.

The following biochemicals and radiochemicals were obtained from the Suppliers indicated.

Amersham International plc, Amersham Laboratories, Buckinghamshire:

$Na^{125}I$ (13.5 mCi · μg^{-1})

L-[U-^{14}C] Glutamine (10.5 GBq/mmol, 285 mCi/ mmol, 1.85 MBq/ml)

L-[U-^{14}C] Glutamic Acid (10.5 GBq/mmol, 285 mCi/mmol, 1.85 MBq/ml)

L-[^{35}-S]-Methionine (1000 Ci $mmol^{-1}$)

γ^{32}-P ATP

α^{32}-P GTP

B.D.H. Chemicals Ltd., Poole, Dorset:

Acrylamide, ammonium persulphate, sodium dodecyl sulphate.

Eastman Kodak, Rochester, NEW York, USA;

N,N'-methylene bisacrylamide, N,N,N,N,'-tetramethylene diamine.

Pharmacia (GB) Ltd., London:

Sephacryl S 300 superfine, Blue Sepharose, Sephadex-G 50,

Low molecular weight protein kit and high molecular weight protein kit for denaturing polyacrylamide gels, molecular weight protein marker for gel filtration column.

Sigma Chemical Co.Ltd., Poole, Dorset:

Agarose (type 1 low EEO), bovine serum albumin (BSA), Coomassie brilliant blue R, phenylmethylsulphonyl fluoride (PMSF), *Staphylococcus aureus* Protein A.

2.3. Methods used in biochemical study of NADH-GOGAT

1. Growth of *Rhizobium*

1). Preparation of Yeast Mannitol media and Agar

K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.2g
NaCl	0.1g
Mannitol	10.0g
Yeast extract	1.0g
Agar	15.0g

NB. solution for rhizobia growth without agar

Add the above components to distilled water and make up to 1 liter. Adjust the pH to ebout 6.8 and autoclave at 120°C.

Make the slope in universal vial with the autoclaved agar solution.

2) Growth of *Rhizobium*

Rhizobium strain was grown on slopes at 30°C for two days and then transferred to 10 ml YM solution and incubated at 30°C overnight and then transfered to 1 liter flask with 500 YM solution and incubated at 30°C for two days. It was then ready for inoculating the plants.

2. Hewitt's -N Nutrient Solution for Nodule Growth

Major nutrients (for 50 liters)

$MgSO_4.7H_2O$	18.4g
$Na_2HPO_4.12H_2O$	23.9g
K_2SO_4	17.4g
$CaCl_2.6H_2O$	43.8g

Dissolve each separately in 400 ml of deionised water.

Trace element stock solutions

$MnSO_4.4H_2O$	1.115g
$CuSO_4.5H_2O$	0.25g
$ZnSO_4$	0.145g
H_3BO_3	1.55g
NaCl	2.925g
$(NH_4)_6\ Mo_7O_{24}.4H_2O$	0.044g
$CoSO_4.6H_20$	0.265g

Dissolve each separately in 100 ml of deionised water

NaFeEDTA 18.35g/500ml deionised water

Mix trace elements (1ml of each, except for 2.5ml of NaFeEDTA) in a 2 liter flask half filled with tap water. Add 40ml of each stock solution except $CaCl_2$. Add 1.5ml of 2N HCl. Add $CaCl_2$ mixing quickly. Make up to 5 liter with tap water. Adjust pH to ~6.5.

3. Preparation of nodule samples

1).Preparation of plant-cell free extract

Frozen nodules (0.5 g) or root system (2 g) were ground in a mortar and pestle with 2.5 ml extraction buffer (50 mM HEPES pH 7.5 containing 0.5 M sucrose, 10 mM DTT, 1 mM EDTA and 1mM PMSF). The homogenate was transferred to microfuge tubes and centrifuged at 11,600 x g for 10 min. The supernatant (about 2.5 ml) was desalted on a 5 ml Sephadex G-50 column and either used directly or prepared for the HPLC column as follows. The extract was diluted to 6 ml with HPLC running buffer (50 mM potassium phosphate buffer pH 7.5, containing 1 mM EDTA and 1 mM DTT) and filtered through a 0.2 µm filter and used immediately for enzyme activity measurements and chromatography on HPLC column. All procedures were carried out at 0°to 4°C.

2).Preparation of nodule samples for localization of NADH-GOGAT two isoenzymes

A. Nodule samples for sucrose gradients

Freshly picked nodules (10 g) were gently pressed until broken with a spatula spoon in 20 ml 0.1 M Tricine buffer, pH8.0, containing 0.4M sucrose, 10mM KCl, 10 mM EDTA, 5 mM DTT, 1 mM $MgCl_2$, 1mM PMSF and 2mM reduced glutathione, and filtered through Miracloth to separate inner nodule tissue from outer cortex tissue. The pooled extract of the inner

tissue

A).was applied directly to a step gradient described in 2.5.A. by Boland et al., 1982 to isolate mitochondria, plastids and bacteroids.

B).was centrifuged at 1650 x g for 4 min to pellet cell debris and starch granules and gave a light brown sloppy layer which contained the bacteroids and some proplastids. The darker red fluid above the bacteroids was carefully poured off and kept for centrifugation on sucrose gradient to prepare plastids as described in (Boland and Schubert, 1983).

B. Nodule samples for isolation of outer cortex:

Freshly picked nodules (about 5 g) were gently pressed with a spatula spoon in 50 mM pH 7.5 HEPES buffer containing 10mM DTT, 1mM PMSF, 0.5 M sucrose at 0°C. The inner part constituents were washed off with the above buffer until the outer cortex was white. The outer cortex was ground with a pestle in a mortar with 2 ml of the above buffer and transferred into a microfuge tube and centrifuged for 5min in cold room. The supernatant was diluted 4-fold and then loaded onto HPLC ion-exchange column.

4. Isolation and identification of two NADH-GOGAT isoenzymes

1) Chromatography on HPLC ion-exchange column

5ml of desalted extract was loaded onto a DEAE TSK 5PW (7.5 x 75 mm) column preequilibrated with HPLC running buffer at room temperature. The proteins were then eluted at a flow rate of 0.5 ml min^{-1} with 5 ml HPLC running buffer followed by 20 ml of a 0 to 0.4 M KCl gradient and then 5 ml of 0.6 M KCl all made up in HPLC running buffer. 0.5 ml fractions were collected, placed on ice and then assayed for NADH-GOGAT activity.

2) Chromatography on HPLC gel filtration column

0.5 ml of extract was loaded onto an analytical HPLC gel filtration column (TSK G4000SW 7.5 x 600 mm) preequilibrated and then run with the buffer described above. 0.5 ml fractions were collected at a flow rate of 0.5 ml min^{-1}. Marker proteins dissolved in the same buffer were run under identical conditions; the proteins used were β-galactosidase (M_r 520,000), glutamate dehydrogenase (M_r 340,000), catalase (M_r 232,000), alcohol dehydrogenase (M_r 150,000), haemoglobin (M_r 64,000), cytochrome c (M_r 13,000).

5. Localization of two NADH-GOGAT isoenzymes: separation of the organelles on sucrose gradients

1) Preparation of sucrose gradients and centrifugation for isolation of different organelles

A. Isolating mitochondria, plastids and bacteroids followed the method described by Boland *et al.*(1982)

2.5ml of 2.3 M sucrose in 0.1 M Tricine-KOH pH7.8 buffer containing 2mM DTT, 2 mM reduced glutathione and 10 mM KCl was pipetted into a 38 ml cellulose-nitrate tube at 0° to 4°C. 34 ml of linear gradient of 0.75-2.0 M sucrose in the above buffer was then gently prepared on top of the 2.3M sucrose. Finally 3ml of sample was gently loaded on the top. All gradients were centrifuged in a Beckman SW 27.2 rotor at 4°C at 11,000 rpm (16,000 g) for 4.5 h. after slow acceleration. After the run, 2ml fractions were picked out with an automatic pipette.

B. A rapid method for isolating plastids (Boland and Schubert 1983)

23ml of 2M sucrose in 0.1M Tricine-KOH pH 8.0 buffer containing 2mM DTT, 2mM reduced glutathione, and 10mM KCl was pipetted into a 38ml cellulose-nitrate tube and 12ml of 0.8 M sucrose in the same buffer was then gently loaded on the top of the 2 M sucrose. Finally, 3ml of nodule sample from 2.2.2 B. was carefully layered on the sucrose gradient and was then centrifuged at 11,000 rpm (giving a rcf range from

14,000 to 19,000 x g over the length of the gradient), for 30 min in a swinging bucket rotor SW27 at 4°C. The pellicle, about 2 ml, at the interface between the two sucrose layers was withdrawn with a syringe. This fraction was referred to as the plastid fraction. The fluid remaining at the top of the gradient was considered to be the soluble fraction from the nodule.

6. Purification of two NADH-GOGAT isoenzymes

1) Crude Extract

All the procedures were carried out in a cold room except steps 4 and 5.

100 g of frozen nodules were ground in a coffee grinder for 2 min then transferred to a mortar and ground with pestle in about 200 ml extraction buffer. The brei was then diluted to 500 ml with extraction buffer, filtered through four layers of muslin and centrifuged at 11,000 x g for 20 min. The supernatant was then subjected to stepwise ammonium sulphate precipitation and the pellet, precipitated by 35 to 60% saturation with ammonium sulphate, was dissolved in 20 ml of potassium phosphate buffer pH 7.5 containing 1 mM PMSF.

2) Gel filtration on Sephacryl S-300

The extract was loaded onto a 100 x 3.5 cm Sephacryl S-300

column. The column was run overnight with 50 mM potassium phosphate buffer containing 1 mM EDTA and 0.5 % β-mercaptoethanol (running buffer) at 24 ml h^{-1} and 8 ml fractions were collected. Fractions showing highest activity of NADH-GOGAT were pooled and glutamic acid (Na salt) was added to 5 mM.

3) Chromatography on Blue Sepharose column

The partially purified extract was then loaded onto a 30 x 1.5 cm Blue Sepharose column previously equilibrated with running buffer. The column was washed overnight with about 400 ml of the same buffer and then with 100 ml of the same buffer but containing 0.1M NaCl. NADH-GOGAT activity was then eluted with running buffer containing 0.3M NaCl, 50 mM 2-oxoglutarate and 50mM L-glutamine. The fractions containing NADH-GOGAT activity were pooled and solid ammonium sulphate was then added to 80 % saturation. The precipitated proteins were collected by centrifugation and the pellet was dissolved with 2 ml HPLC running buffer and desalted on a Sephadex G-50 column (as above).

4) Chromatography on HPLC Ion-Exchange Column

The desalted extract was made up to 5 ml and chromatographed on the HPLC ion-exchange column as described above.

5) Chromatography on HPLC Gel Filtration Column

The separated two isoenzymes were purified to homogeneity by

passage through an HPLC gel filtration column (as above).

7. Denaturing polyacrylamide gel electrophoresis

The proteins after each purification step were run on a polyacrylamide SDS gel (Laemmli, 1970) with a discontinuous buffer system, using the LKB 2001 Vertical Electrophoresis Unit.

1)The following were stock solution used:

A. 30%(W/V) acrylamide

B. 2%(W/V) bisacrylamide

C. 10%(W/V) SDS

D. 1M Tris HCl pH8.8

E. 1M Tris HCl pH6.9

15.0% gels, containing the following

A 30ml

B 2.6ml

C.0.6ml

D.22.4ml

Water 4.25ml

10.0% gels, containing the following

A 20ml

B 4ml

C.0.6ml

D.22.4ml

Water 12.85ml

Stacking gel

A. 1.7 ml

B.0.7ml

C.0.1ml

E.1.25ml

Water 6.25ml

2).The electrode buffer

Per liter

SDS	1.0g
Tris base	6.0g
Glycine	28.8g

The gel was run at 30 mA constant current for $4^1/_2$ hours.

3).Sample buffer

1 M Tris·Cl pH 6.8	1ml
10% (W/V) SDS	2ml
20% (V/V) glycerol	2ml
4% BPB	100µl
H_2O	5ml

Before running the gel, take 200 µl of above mixture and add 4 µl of β-ME as sample buffer. The samples were mixed with the equal volume of sample buffer. Once in sample buffer, all samples were heated to 100° C for 3 min. prior to loading.

4). Autoradiography and staining gels

The electrophoresed gels, or portion of them, were either left unfixed and immediately prepared for Western blotting of proteins onto nitrocellulose (described elsewhere) or fixed in 50% methanol with 3-5 times changing for silver staining or gels were stained with Coomassie blue solution containing methanol and acetic acid directly. Gels containing ^{35}S-methionine labelled proteins were fixed in 45% (V/V) methanol, 10% (V/V) acetic acid for 30 min and then were fluorographed with Amplify for 30 min., after that gels were dried down for autoradiography.

Molecular weight markers were myosin (M_r 200,000), β-galactosidase (M_r 116,250), phosphorylase B (M_r 92,500), bovine serum

albumin (M_r 66,200), ovalbumin (M_r 45,000).

8. Protein electroelution procedure (Laemmli, 1970)

1).Acrylamide gel was stained very briefly with Coomassie blue (5-10 min.), then rinsed in destain until the protein bands became visible (5-10min.). The staining and destaining times were kept as short as possible for maximum yields.

2). The flat-bed tank was filled with buffer (Electrophoresis buffer with 2 mM DTT).

3). The band of interest was cut off and put into a dialysis bag with the buffer from the tank, so that the amount of the buffer just covered the gel slice. The two ends were clipped with mediclips and care was taken to avoid trapping any air bubbles. The gel slice was positioned on one side of the dialysis bag.

4). The dialysis bag was placed onto the platform of the electrophoresis tank. The level of the buffer was adjusted so that it just covered the bag.

5). Electrophoresis was carried out for about 20h. at 20 mA constant current then the current was reversed for approx 30 seconds.

6). The gel slice was removed from the bag and discarded. The solution was taken to a microfuge tube and centrifuged briefly to pellet any small pieces. The solution was put back into the dialysis bag and the protein solution was dialysed against at least 5 changes of distilled water (4-5 liters each) over 2-3 days at 0°-4°C.

7). The dialysed protein solution was frozen and lyophilised.

9. Enzyme assays

1) Assay of NADH-GOGAT Activity

A. Spectrophotometric assay.

The activity of NADH-dependent GOGAT was assayed at 18° to 22°C by measuring the oxidation of NADH at 340 nm. The reaction mixture routinely contained 25 mM HEPES buffer pH 7.5, 1 mM 2-oxoglutarate, 2.5 mM L-glutamine, 0.16 mM NADH, 1% β-mercaptoethanol and 100-200µl enzyme solution in a total volume of 1.2ml. The reaction was initiated by the addition of the enzyme and the change in A_{340} (E_{340}= 6.22x 10^6 cm^{-1} mol^{-1}) was monitored over a period of at least 2 min and was found to be linear over the time. An assay without 2-oxoglutarate and L-glutamine was used as control. The activities are expressed in µmol NADH oxidized min^{-1}. In the determination of the Km values of NADH-GOGAT for the various substrates, the concentration of

the substrates were varied in the reaction mixture. For pH determinations the pH of the HEPES buffer was varied. Partially purified extracts prepared as in the purification procedures but lacking the Blue Sepharose and final gel filtration steps were used in these determinations.

B. Radiochemical assay.

NADH-GOGAT activity was measured by the production of ^{14}C-labelled glutamate from ^{14}C-glutamine essentially as described by Wallsgrove *et al.* (1982) with some modifications. ^{14}C-glutamine, obtained from Amersham International, was purified on a column of Dowex-1-chloride and was used at a specific activity of 109 μCi/mmol in the reaction mixture. The separation of glutamate from glutamine by paper electrophoresis was achieved at about 90V for about 4h.

2). Assay of Fd-GOGAT Activity

Fd-GOGAT activity was measured according to the method described by Wallsgrove *et al.* (1982) but with the modifications as mentioned above.

3). Assay of Nitrogenase Activity

Nitrogenase activity of nodulated root systems was measured by acetylene reduction as described previously (Dart *et al.*, 1972).

The sample consisted of top 5 cm root system was put in a

universal bottle covered with a Subaseal through which 100 µl of acetylene was injected to give an acetylene concentration of about 3.3%. After shaking, the bottle was incubated in the light. Within 1h, 100µl of mixture of the gases in the containers was taken every 5 min by Becton Dickinson plastipak syringe and then injected into the gas chromatograph and C_2H_4 was quantified by a flame ionisation detector. The colume packing system used was 80-100 mesh Poropak R in a 1m x 0.003m diameter glass column at 100°C, with a nitrogen carrier gas flow rate of 20ml/min, using a hydrogen/air flame ionisation detector.

After assay, the nodules were picked off the root and weighed .The nitrogenase activity is expressed as µmols ethylene produced per min per gram fresh weight nodule.

10. Protein determination

Protein, in cell-free extracts, was determined using the BioRad Protein Assay Dye-Binding reagent, and bovine γ-globulin as standard. Protein standard containing 4 mg/ml γ-globulin was prepared and a standard curve was performed for each assay. 10-100 µg of standards and 100µl of appropriately diluted samples were put into microfuge tubes and made to 200µl with assay buffer. 1.0ml diluted 5-fold dye reagent was added to each tube. After mixing, A_{595} was measured versus reagent blank. A_{595} versus concentration of standards was plotted. Unknowns were read from the standard curve (Fig 2.4.).

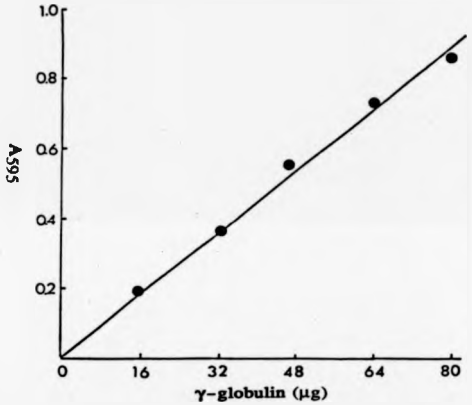


Fig.2.4. Standard Curve For the Bio-Rad Protein Assay

11. Marker proteins assay

For determination of NADH-GOGAT molecular weight, the HPLC gel filtration column (TSK G 4000SW 7.5x 600mm) was calibrated using a number of globular proteins. The column was preequilbrated with HPLC running buffer. Marker proteins dissolved in the same buffer were run under identical conditions. The marker proteins were assayed as described below followed the methods described by Cullimore (1980) and their activity or abundance expressed in arbitrary units/fraction.

1).β-galactosidase (E.C. 3.2.1.23.-from *E.coli* M_r 520,000).

The reaction mixture contained in 0.5ml: 25 µmol sodium phosphate buffer, pH 7.5; 0.25 mg o-nitrophenyl-β-D-galactopyranoside; enzyme extract. The reaction was initiated by addition of enzyme and terminated by adding 0.5 ml 4% Na_2CO_3. The absorbance due to o-nitrophenol was measured at 400 nm.

2).Glutamate dehydrogenase (E.C. 1.4.1.3. from bovine liver M_r 340,000)

The reaction mixture contained in a final volume of 1.0 ml in 50 mM pH9 Tris buffer, 10 mM L-glutamate, 3µmol NAD, 1mM $CaCl_2$, 7mM $MgCl_2$. The reaction was initiated by addition of enzyme and activity determined by following NAD reduction at 340 nm.

3).Catalase (E.C. 1.11.1.6. –from bovine liver. M_r 232,000)

The reaction mixture contained in 1.0 ml: 50 µmol sodium phosphate buffer, pH 6.8; 2.5 µl H_2O_2; enzyme extract. The assay was initiated by addition of enzyme and activity was determined by following the disappearance of H_2O_2 at 225 nm.

4).Alcohol dehydrogenase (E.C. 1.1.1.1. – from yeast. M_r 150,000)

The reaction mixture contained in 1.0 ml: 50 µmol Imidazole; 1% ethanol; 0.5 mg NAD; enzyme extract. The reaction was initiated by addition of enzyme and activity determined by following NAD reduction at 340 nm.

5).Haemoglobin (M_r 64,000)

Haemoglobin was estimated by its absorbance at 430 nm.

6).Cytochrome c (M_r 18,500)

Cytochrome c was estimated by its absorbance at 550 nm.

12. Marker enzymes assay for localization of two isoenzymes of NADH-GOGAT

For determination of which organelle is which, the following marker enzymes of different organelles were assayed.

1). β-Hydroxybutyrate dehydrogenase (E.C.1.1.1.30.): marker enzyme for bacteroids (Hanks, 1981).

The rate of reduction of NAD at 340nm was followed on a spectrophotometer using an assay mixture of 3.0 mM $MgCl_2$, 1.2 mM NAD^+, 20 mM sodium-DL-β-hydroxybutyrate and made up to volume (3mls) with 100 mM Tris-Cl buffer (pH 8.0). 100 µl of the extract was used for each assay.

2). Phosphoglycerate Dehydrogenase (E.C. 1.1.1.95.): marker enzyme for plastids (Boland *et al.*, 1983).

Assay was carried out in 0.1 M K-phosphate buffer, pH 7.5, containing 50 µm NADH and 50 µl enzyme in a volume of 0.98ml. The rate of background oxidation of NADH was measured spectrophotometrically at 340 nm; then 20 µl of 20 mM P-hydroxypyruvate was added and the substrate-dependent rate of NADH oxidation was measured. The difference

in the rate was considered to be the result of P-glycerate dehydrogenase activity.

3) Triosephosphate isomerase(E.C.5.3.1.1.):marker enzyme for plastids

Triosephosphate isomerase was assayed by the method of Gibbs and Turner (1964). The assay mixture contained 0.1mM NADH, 10mM DL-glyceraldehyde-3-phosphate, 0.1ml 2units/ml glycerolphosphate dehydrogenase and made up to 1ml with 0.5M Tris buffer, pH 7.5. 100µl of the collected extract was then added and the change in A_{340} was recorded over a period of 2min at 30°C.

4). Fumarase (E.C.4.2.1.2.):marker enzyme for mitochondria

Assay was carried out in 3 ml 0.1M pH 7.5 potassium phosphate buffer containing 50 mM malate. After adding 20 µl of enzyme, the rate of disappearance of malate was measured on a spectrophotometer at 240nm.

13. Immunological techniques

1) Production of Antibodies

NADH-GOGAT from Sephacryl S-300 column and Blue Sepharose chromatography was precipitated by 80% $(NH_4)_2SO_4$, the pellet was

dissolved in HPLC running buffer containing 1 mM PMSF and then was desalted through Sephadex G-50. The desalted enzyme was purified further by SDS-PAGE. The enzyme was then electroeluted as described above and used in the immunisation of rat. Rats were hypodermic injected with between 20μg and 40μg of NADH-GOGAT protein each time. In the first injection the purified proteins were mixed with an equal volume of Freund's complete adjuvant (Grand Island Biological Co. Ltd.) while subsequent injection contained incomplete adjuvant. Before the third injection, blood was taken to test the production of antibodies. Each rat received 8 booster injections at fortnight intervals. Blood was collected from cutting the tail and for the last time, the rat producing the antibodies was terminately bled by cardiac puncture. Blood was allowed to clot at room temperature, serum was removed by pipette and centrifuged at 3,000 xg for 30minutes at 4 °C to remove red blood cells. Antiserum raised against NADH-GOGAT was stored at -20°C with no further treatment.

2).Ouchterlony immunodiffusion

Sera were tested by the double immunodiffusion technique as described by Cullimore and Miflin (1983). A 1% (W/V) solution of agarose in phosphate buffered saline (PBS) was prepared by heating to 100°C. The agarose was allowed to cool to 50°C and the gels were cast on a petri dish about 3mm thick. Six or five wells (diameter 4mm) were punched around a central well.

A. The titer of the antiserum

The serum under test was diluted into 1/2, 1/4, 1/8,1/16, and the diluted serum were placed in the surrounding wells. Nodule extract was placed in the central well.

B. The organ specificity

The serum (10μl) was placed in the central well and the extracts from different organs of *Phaseolus vulgaris* and nodule extract from soybean was placed in the surrounding wells. The petri dish was incubated overnight at room temperature in a closed box containing moist filter paper. After incubation the gels were washed in PBS overnight and stained by immersion in the following solution:

0.25% (w/v) Coomassie brilliant blue R
50% (v/v) methanol
7% (v/v) acetic acid

Excess stain was removed by washing gels in 40% (v/v) methanol; 7% (v/v) acetic acid. The gels were then photographed.

3). Identification of antibody by immunoprecipitation of enzyme activity.

A. 0, 5, 10, 15, 20μl of antiserum were incubated on ice for 90min. with

100 µl of extract separately in 100mM K-phosphate buffer pH 7.8 containing 1mM PMSF, 10mM DTT, 1mM EDTA.

B. At the same time, five aliquots of 50µl of Sepharose proteinA (143mg/ml PBS) were incubated on ice with 100µg of rabbit anti-rat IgG in 100 mM K-Phosphate buffer as mentioned above.

C. Each sample from A was mixed with each aliquot from B and the mixture was left on ice for 90 min.

D. The mixture from C was centrifuged in microfuge at 4°C for 10 min. The supernatant was assayed for NADH-GOGAT activity.

E. If there was no NADH-GOGAT activity in supernatant, Sepharose beads from D. were washed with K-Phosphate buffer as metioned in StepA containing 2%triton for 4 times followed by adding 100µl of PAGE loading buffer and boiling for 4 min and then were run on SDS-polyacrylamide gel.

4). Analysis of proteins by immunoblotting ("Western" Blotting)

Adapting previously published methods (Gershoni and Palade, 1983), all blotting was performed using the BioRad Transblot apparatus.

Samples to be examined were subjected to SDS polyacrylamide gel electrophoresis as described in 2.2.8. After electrophoresis, the stacking gel was cut away with a scalpel and the remainder of the gel trimmed if

necessary. The gel was then washed in "Western transfer buffer" of the following composition:

192 mM glycine
25 mM Tris base
20% (v/v) methanol
0.1 (w/v) SDS

The gel was placed on a piece of foam ("Scotch-brite") and a piece of nitrocellulose (pore size 0.45μm) was cut to fit the gel exactly. The nitrocellulose was soaked in "Western" transfer buffer and laid over the gel, care being taken to avoid bubbles between layers. A second "Scotch-brite" pad was placed over the nitrocellulose.

Transfer was performed as described by Vaessen *et al.* (1981). The gel sandwich was placed in a Trans-Blot Cell (BioRad) which contained 3 liters of transfer buffer. Transfer was performed at 4°C for 20 h. at 45 volts with stirring. After transfer, filters were incubated with shaking in 100 ml of PBS containing 3% Marvel (W/V), over 2h with three changes of solution to saturate unbound sites.

A. Western blot for peroxidase colour development

After saturating unbound sites, the filter was developed either by peroxidase colour development or by ^{125}I-protein A labelling method.

For peroxidase colour development, the filter was incubated with

25-100 µl of antiserum in 10ml of 0.1% Tween/PBS and was sealed in a plastic bag either for 4h or for overnight with shaking at 200 rpm at 30°C. After washing 5 times in 0.1% Tween/PBS over 1h, the filter was incubated with 33µl of Biotinylated Protein A in 10 ml of PBS in a sealed plastic bag with shaking at 30°C for 1h. After washing 5 times with 0.1% Tween/PBS, the filter was incubated with 33 µl of Streptavidin Peroxidase in 10 ml of PBS same as previous step for 30 min. After washing twice with 1% Tween/PBS and twice with PBS the filter was incubated with immuno staining solution containing mixture of solutionA and solutionB:

Solution A : 1.46g NaCl in 50ml 20 mM Tris ·Cl buffer with 30 µl of 30% H_2O_2.

Solution B: 30mg 4-Chloro-1-Napthol in 10 ml of methanol.

Once blue protein bands appeared, the developing reaction was stopped by washing the filter in water repeatedly over 60min and the filter was dried in the dark and then was photographed.

B. Western Blotting for ^{125}I-Protein A Labelling

After saturating remaining unbound sites by incubating with Marvel, the filter was incubated with 25-100 µl of antiserum in PBS overnight followed by washing the filter 6 times with 100ml of PBS and incubating with 3% Marvel dissolved in PBS containing 10^6 cpm [^{125}I]-protein A for 3h. All unbound protein A was removed by washing

with PBS containing 1% (V/V) Triton-X 100 several times. The filter was then dried and autoradiographed at -80°C using an intensifying screen as described in 2.2.8. Positions of radioactive bands on "Western" blots were determined from the corresponding autoradiograph.

2.4. Methods for the cloning of gene(s) encoding NADH-GOGAT

1.Growth and storage of bacteria:

For long term storage of bacteria (over 9 months), bacteria were kept as frozen glycerol stocks. 500µl of a 10 ml overnight culture in LB (10g bactotryptone, 5g yeast extract, 10g NaCl, pH 7.5 per liter ddH_2O) mixed with 300µl of 50% glycerol and stored frozen at -70°C.

2.Agarose gel electrophoresis

1). Rapid analysis agarose gel electrophoresis

For rapid analysis of restriction enzyme digest products or DNA quantitation, a mini-gel tank (Uniscience) was used; 50 ml of 0.7%(w/v) agarose in 1xTBE (1 liter of 10 x TBE consisted of Tris Base 108g, Boric acid 55g Na_2EDTA 9.3g, pH 8.3) was prepared by melting the

agarose in the buffer using a microwave oven. The molten gel was cooled to 60°C and EtBr added to 0.1mg/ml. The gel was poured into the gel tank containing either an 8- or 16-place comb and left to set for 30min at room temperature. The comb was removed and the gel covered in 1xTBE buffer. 5x loading buffer was added to each sample. The samples were loaded into the wells and the gel run for 30-60 min at 70 mA. Gels were visualised and photographed.

2). Alkaline agarose gel electrophoresis

Alkaline agarose gels (McDonnell *et al.* 1977) was used to check the size of the first and the second DNA strands synthesized by reverse transcriptase. Because the addition of sodium hydroxide to hot agarose solution causes hydrolysis of the polymer, the gel was prepared in a neutral, unbuffered solution (50mM NaCl and 1mM EDTA) and equilibrated in alkaline electrophoresis buffer (30mM NaOH, 1mM EDTA) before running. For a 1.2% 100ml gel, 1.2g agarose was melted in 150ml 50mM NaCl and 1mM EDTA in a microwave oven and was cooled to 50°C and ethidium bromide (10mg/ml in water, stored in a light-proof bottle) was added to a final concentration of 2μg/ml. The gel was poured into a mid-size plastic plate with sealed edges with tape. A 10-teeth comb was placed and the gel was set at room temperature for about 30min. After the gel was completely set , the comb was removed carefully and the running buffer containing 30 mM NaOH, 2 mM EDTA was added to the tank to cover the gel. 1μl (0.1μCi) of the first strand cDNA, 1μl (0.1μCi) of double strand cDNA,

and 4µl of marker DNA pBR332/*Hae*III, λDNA/*Acc*I, were mixed with 5-10 µl of loading buffer (200µl of 20% glycerol, 10µl of 4% bromophenol blue, 800µl of alkaline /EDTA running buffer). All samples were loaded on the gel immediately and run at 45mV overnight. Then the gel was dried under vacuum and autoradiographed.

3). Formamide agarose gel

SP6 transcription product was run on formamide agarose gel to check the size.

Solutions:

10 x Mops buffer 0.2M Mops

0.05M Sodium acetate

0.01M EDTA

pH was adjusted to 7.0 with NaOH. After autoclaving, the solution turned yellow.

Filtered formaldehyde:

A commercial formaldehyde solution (40% w/v formaldehyde) was filtered through Whatman No.1 paper, which removed any paraformaldehyde that might have precipitated out of solution.

Deionised formamide

Formamide was stirred with BDH 'Amberlite' monobed resin MB-3 till it was pH7.0. Then formamide was filtered through Whatman No. 1 paper in a buchner funnel by a vacuum pump to remove the resin.

Deionised formamide was stored in 500 µl aliquots in microfuge tubes at -20°C.

Procedures:

For a 1.5% 100ml gel, 1.5g agarose was melted with 75ml H_2O and 10ml 10 x Mops buffer in a microwave oven and was cooled to 60°C followed by adding 15ml of filtered formaldehyde and mixing. The gel was poured as described for alkaline agarose gel onto a plastic plate cleaned with ethanol. Because of formaldehyde fumes (toxic, possibly carcinogenic), the gel should be poured in the fume hood. The gel was run submerged in 1 x Mops buffer (the tank requires 1100 ml of running buffer). RNA samples were suspended in sterile H_2O to give about 10µg of total RNA in 5 µl (About 60ng of RNA in a single band is just visible after EtBr staining) and were denatured by adding 15µl of freshly made denaturing solution (To a 500µl aliquot of deionised formamide add 100ml of 10 x Mops buffer and 150ml of filtered formaldehyde) and heated at 60°C for 5min. The RNA samples were cooled on ice and then were put at R.T. RNA sample was added to 2µl of loading mix (50% glycerol, 0.2% bromophenol blue) and was loaded on the gel immediately. Before loading the samples, the wells were flushed out with buffer to remove formaldehyde which affected banding. The gel was run at 100V (about 40mA) for 5-6h. until the dye was 2cm from the end of the gel (5S RNA runs just in front of the dye, the gel could also be run for 2.5 h. to give tighter bands). After the gel was dried under vacuum, the gel was autoradiographed.

For Northern Blotting, the gel was not dried but soaked in 250ml of 10% glycine for 20 min (without this EtBr does not stain the RNA) then 100 µl of 10mg/ml EtBr was added and stained for 10 min. The gel was destained with two changes of H_2O for 15 min. each. After taking

the photograph, the gel was soaked in 250ml of 20 x SSC (3M NaCl and 0.3M Na_3Citrate, pH to 7.0 with HCl/NaOH) for 20min and was ready for Northern Blotting.

3. Plasmid preparation

1). Large scale plasmid preparation (Alkaline hydrolysis method)

Plasmids were isolated from *E.coli* by a modification of the alkaline lysis method of Birnboim and Doly (1979). Ten ml of LB (10g bacto-tryptone, 5g yeast extract, 10g NaCl, pH7.5 per liter of distilled water) containing the appropriate antibiotic in a 25ml universal containers were inoculated with single bacterial colonies from fresh LB agar plates. Cultures were grown overnight in an orbital shaker at 200 rpm and 37°C. Two liter flasks containing 1 liter of LB plus antibiotics were inoculated with the 10ml overnight cultures and grown at 37°C with shaking to an A_{590} value equal to 0.8 units. Plasmid DNA was amplified by the addition of either chloramphenicol to 175μg/ml or, in the case of chloramphenicol-resistant plasmids, with spectinomycin to 300 μg/ml. The flasks were shaken at 37°C for a further 16h. Cells were collected by centrifugation at 2500 rpm and 4°C for 30min in an MSE 6x1000 rotor. The cells were resuspended in 3.2 ml of ice-cold GET (50mM-Glucose, 10mM-EDTA, 25mM-Tris-HCl pH8), transferred to 50ml MSE Oakridge centrifuge tubes

and placed on ice. Cell lysis was obtained by the addition of 200 µl GET containing lysozyme at 40mg/ml and incubation on ice for 10min; 6.6ml of 0.2 M NaOH, 0.1% (w/v) SDS was added to each tube and the tubes gently swirled. After 10 min on ice, 5ml 3M sodium acetate pH 4.5 was added and the contents of the tubes gently mixed. The tubes were left on ice for a further 30 min. After this incubation the tubes were centrifuged at 10,000 rpm and 4°C for 15 min in an MSE 8x50 rotor. Nucleic acid was precipitated by the addition of 8.3 ml isopropanol to the supernatants in sterile 50 ml tubes. Following mixing, the tubes were left at room temperature for 10 min. The precipitates were pelleted by centrifugation at 10,000 rpm and 4°C for 10 min. The pellets were drained and resuspended thoroughly in 3.2ml 2M ammonium acetate and the samples respun at 10,000 rpm for 10min as above. After centrifugation, 2.1 ml isopropanol was added to each sample in sterile 50 ml tubes. After 10 min at room temperature the resulting precipitates were collected by centrifugation as above. The pellets were washed twice in 5ml 70% (v/v) aqueous ethanol. The plasmid pellets were gently dried *in vacuo* and resuspended in 20 ml TE (10mM Tris-HCl pH 8, 1mM EDTA).

Plasmid DNA was further purified by CsCl density gradient centrifugation (Maniatis *et al.*, 1982). CsCl (23.76 g) was gently dissolved in each of the 20ml plasmid solutions. EtBr (4ml of 5mg/ml) was added to each sample and the resulting solutions loaded into 37 ml Beckman Quickseal tubes. The tubes were topped up with either liquid parafin or 50% CsCl in TE buffer, balanced and sealed. The gradients were formed by

centrifugation in a Beckman VTi 50 rotor run at 45,000rpm and 20°C for 18h. The rotor was brought to rest without the aid of brake. Plasmid bands were visualised under long-range u.v. and withdrawn from the decapitated tubes in a volume of under 2ml with 18 gauge needle. The EtBr was removed from the samples by several extractions with butanol alcohol saturated with TE. The plasmid DNA was precipitated with 2 volumes of ethanol and 1/20 volumes of pH 4.5 4M NH_4Ac at -20°C overnight. The plasmid DNA solution was centrifuged at 10,000 rpm for 30 min. The pellet was washed with 70% ethanol and desiccated to dryness and dissolved in 200µl TE buffer. 1µl of plasmid DNA was measured at OD260 and OD280 (or scan from 200 to 300nm) to check the concentration and the purity. The concentration and the purity was further determined by running 1µl of plasmid DNA on mini-agarose gel. The plasmid DNA was stored at 20°C. A typical preparation yielded 500-1000µg DNA.

2). Small scale plasmid preparation

Small scale plasmid preparations were performed using a modified alkaline lysis protocol of Birnboim and Doly (1979) on 2ml overnight cultures. The cultures were grown in LB containing the appropriate antibiotic. The cells were collected by centrifugation and resuspended in 100ml GET (50mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA). Resuspended cells were transferred to 1.5ml microfuge tubes and lysed by the addition of 20 µl GET containing lysozyme at 10 mg/ml and incubation on ice for 10min; 200 µl of 0.2M NaOH, 0.1% (w/v) SDS was added to the tubes and gently mixed. Following 5min on ice 100 µl of 3M

sodium acetate pH 4.8 was added and the lysed cells were incubated on ice for 10 min. Then cell debris and chromosomal DNA was removed by centrifugation for 3min. The supernatants were poured into new tubes and deproteinated by the addition of 200 µl TE saturated phenol and 300 µl chloroform/iso-amyl alcohol (24/1 v/v). The tubes were vortexed and spun in a microcentrifuge for 3min. This step was repeated on the aqueous phase followed by extraction with 500 µl di-ethyl ether. RNA was removed from the samples by the addition of 5 µl boiled RNAase A (1mg/ml). The plasmid DNA was precipitated by the addition of 1/20 volumes of pH 4.5 4M ammonium acetate, two volumes of ethanol and incubation on dry-ice for 15min. The DNA was collected by centrifugation at 4°C for 10min in a microcentrifuge and the pellet washed twice in 70% (v/v) aqueous ethanol. The washed pellet was dried *in vacuo* and resuspended in 50µl of such a preparation was sufficient for analysis on an agarose gel.

4. Isolation of DNA following electrophoresis

1). Elution of DNA from agarose gels

This method is a modification of that used by Dretzen *et al.* (1981) and was found to give very high yields of DNA that was essentially free from any inhibitors of DNA restriction and modification enzymes. DNA fragments to be isolated were subjected to electrophoresis through 1x

TAE agarose gels. A slot was cut in front of the desired band and a piece of Whatman DE81 paper (pre-soaked overnight in 2.5M NaCl, 20mM Tris-HCl pH 7.5, 1mM EDTA, rinsed 5 times in SDW and stored at 4C in 1mM EDTA) inserted into the slot. The gel was re-run until the DNA band had bound to the paper. The paper was removed from the gel and placed in 300µl per 50mm2 NTE buffer (1.5M NaCl, 20mM Tris-HCl pH 7.5, 1mM EDTA) in a 1.5 ml microfuge tube, vortexed and incubated at 37° C for 2h followed by a further 10 min at 50°C. The paper was pelleted by centrifugation at room temperature for 10min. The supernatant was then spun-filtered through two layers of Whatman no.1 paper into a clean microfuge tube. The yield of DNA was increased by spinning a further 50µl NTE buffer through the filter paper. The EtBr was removed from the sample by extration three times with an equal volume of water-saturated butanol. The DNA was concentrated by the addition of two volumes of ethanol and incubation on dry-ice for 15min. The precipitated DNA was pelleted by centrifugation, washed twice with 70% ethanol, dried, and resuspended in SDW to a concentration of 50ng/µl.

2. Electro-elution of DNA from agarose gel

In cases where DNA fragments were to be eluted rapidly the fragment was located as described above and the excised gel slice was placed in 400 µl 1xTBE buffer. The buffer and gel slice were placed in EDTA-treated dialysis tubing. The slice was placed against one edge of the tubing which was sealed at both ends with dialysis tubing clips. This tubing

was subjected to electrophoresis in 1xTBE buffer for 30min at 150v in a mini-gel tank, the polarity was reversed for 10s and the DNA solution removed to a 1.5ml microfuge tube and was extracted with an equal volume of phenol, then half-volume of phenol and a half volume of chloroform/iso-amyl alcohol (24/1, v/v), re-extracted with an equal volume of phenol. The DNA was precipitated by the addition of 1/10 volume 4M NH_4AC, pH 4.5 and two volumes ethanol at -20°C overnight. The precipitated DNA was pelleted by centrifugation, washed twice with 70% (v/v) aqueous ethanol, dried, and resuspended in 10 µl ddH_2O.

5. Extraction, purification, and analysis of polyA$^+$ RNA from root nodules of *Phaseolus vulgaris* L.

1) Isolation of total cellular RNA and selection of polyA$^+$ RNA

All procedures were carried out over three days.

On the first day, 39g root nodules stored in liquid nitrogen were ground in a coffee grinder for 1-2 min., then were transferred to a mortar and ground with 70 ml of RNA extraction buffer for 1-5 min in cold room.

RNA Extraction Buffer

200 ml 200 mM Trizma-Cl buffer pH 7.5 containing:

400 mM NaCl

60 mM $MgCl_2$

50 mM Na_2-EDTA

was autoclaved and then the following was added:

5mM DTT

250mM Sucrose (RNAase free)

1% SDS

The mixture was passed through 2 layers of sterilized muslin and then centrifuged at 15,000 x g for 20min in cold room. Supernatants were extracted using phenol and $CHCl_3$ twice. The upper layer was precipitated with ethanol at -80°C for 2hrs. After centrifugation at 25,000 rpm for 30 min, the pellet was washed with 20ml of 75% ethanol and then centrifuged at 2,500 rpm for 15min. The pellet was desiccated and resuspended in 500 µl of sterile ddH_2O (double distilled water) first and then made up to 6 ml with ddH_2O. 150 mg/ml NaCl was added to the solution, dissolved and left at 4°C overnight to precipitate RNA.

On the second day, the suspension containing RNA was centrifuged at 10,000 rpm for 30 min at 0°C. The pellet was dispersed with 3ml of 2.5M NaCl on ice and centrifuged at 10,000rpm for 30min at 0°C.The pellet was treated exactly the same way with 2.5 M NaCl for another 3 times until the pellet was white. The pellet was then washed with 75% ethanol

once, desiccated and then dissolved in 400µl of ddH_2O. Lastly 8ml oligodT-binding buffer (400ml 10mM Trizma-Cl pH 8.5 buffer containing 1mM EDTA, 0.1% SDS, o.4M NaCl) was added to the solution. The solution was heated at 65°C for 5 min and then passed through oligo dT-cellulose column to isolate $polyA^+$ RNA. The oligo dT-cellulose column kept in 0.1N NaOH all the time was washed with 50ml of 0.1N NaOH. Then the column was equilibrated with 50 ml of oligo dT binding buffer. RNA extract was loaded onto the column, pump was set at 1.5ml min^{-1} followed by washing the column with 30ml binding buffer until OD_{260}=0. $PolyA^+$ RNA was eluted with oligo dT elution buffer which is the same as binding buffer except without NaCl. The column was washed with 0.1 NaOH when all of RNA was eluted. The eluted $polyA^+$ RNA was added 1/20 volumes of 4M NH_4Ac (pH 4.5) and was concentrated by adding 2.5 volumes of ethanol at -20°C overnight.

On the third day, the $poly^+A$ RNA suspension was centrifuged at 10,000 rpm for 30 min. at 4°C. The pellet was washed in 10ml 75% ethanol and centrifuged at 10,000 rpm for 30min at 0°C. Then the pellet was desiccated and redissolved in 400µl ddH_2O in a microfuge tube followed by adding 1/20 volumes of 4M NH_4Ac (pH 4.5) and 2.5 volumes of ethanol. The mixture was mixed gently and kept at -80°C for 2h to precipitate the purified $polyA^+$ RNA out. The $polyA^+$ RNA suspension was centrifuged in cold room for 10 min and the pellet was washed with 1ml 75% ethanol and centrifuged repeatedly three times. Lastly the pellet was desiccated and redissolved in 100µl ddH_2O. The purity was estimated by

measuring OD_{260} and OD_{280} of 1µl of polyA$^+$ RNA in 1ml of ddH2O. Pure RNA has an OD_{260}/OD_{280} ratio of about 2. An OD_{260} of 1.0 is equivalent to 40mg/ml RNA. The yield of polyA$^+$ RNA from root nodules was about 2µg/g fresh weight.

2). Poly$^+$A RNA size-fractionation

The procedures followed the method described by Cullimore and Miflin (1983). Linear, 5-20% sucrose gradients (made in 95% DMSF and 4% formamide) was prepared in 5 ml polyallomer tubes with gradient maker. Before loading the sample, one of the gradients was checked with a refractometer. PolyA$^+$ RNA (39µg/51µl), prepared as above but passed twice through the oligo dT-cellulose column, was boiled for 1 min. then cooled rapidly and mixed with the same volume loading buffer (10 mM Tris·Cl pH 7.4 autoclaved buffer containing 10 mM LiCl, 1mM EDTA 10% DMSO). The mixture was layered onto the top of the gradient and was then centrifuged for 37 h. in a SW 50.1 rotor at 40,000 rpm at 28°C. Marker RNAs rRNA and tRNA mixture was also run on another sucrose gradient under the same condition. After centrifugation the gradient was fractionated into 0.2 ml aliquots in microfuge tubes with an automatic fraction collector and the absorbance for each fraction at 260nm was monitored with a spectrophotometer. By comparing with the absorbances of fractions from the marker, fractions 5 to 16 from mRNA sample gradient containing RNA were precipitated separately with ethanol exactly the same as above. The

pellet was desiccated and then dissolved in 10μl of ddH_2O.

3). Analysis of polyA$^+$RNA by cell free translation

The *in vitro* assay for mRNA fractions was performed in the rabbit reticulocyte lysate system according to the Kit Manual from New England Nuclear.

Translation mixture was as follows: (Supplied in Kit)

	vol.(μl)	final conc.
$Mg(Ac)_2$	2 (diluted 8 fold)	1mM
^{35}S-Met	1	1mM (0.8 μCi)
KAc	1	1mM
Cocktail	2 .75	
Rabbit reticulocyte lysate	5	
mRNA	1	

N.B. Cocktail contains spermidine, creatine phosphate, DTT and GTP in HEPES buffer. Concentrations of the components in the cocktail are the proprietary information of NEN.

All of above constituents were mixed first except rabbit reticulocyte lysate and mRNA. Then translation experiments were carried out by adding 1μl aliquot of each gradient fraction or 1μl of total mRNA respectively and 5μl of rabbit reticulocyte lysate and incubating at 37°C for 1h.

1μl of *in vitro* translation products from each reaction was put on the 1cm squares of Whatman No 1 filter. After about 2-3 min. the filters were boiled in 10% TCA for 10 min. and then were washed with cold 10% TCA three times, 75% ethanol twice and ether last. After drying completely, the filters were transferred into the scintillation counting vial and filled with 5ml non-aqueous scintillation solution. After shaking, the counting was performed on LKB Scintillation Counter.

6. Synthesis of cDNA:

The first and the double strand cDNA were synthesized as described by Gubler and Hoffman (1983) except for the second strand cDNA synthesis without *E.coli* DNA ligase.

1). Synthesis of the first cDNA strand

mRNA fraction 19 corresponding to 200,000 Dalton protein (from Cullimore and Miflin 1983) was used to synthesize cDNA. The reaction was carried out in 30μl of reaction mixture system containing 50mM Tris.HCl pH8.3, 10mM $MgCl_2$, 10mM DTT, 4mM Na pyrophosphate,

1.25mM dCTP, 1.25mM dATP, 1.25mM TTP, 0.5mM dGTP, 15μCi of [α-^{32}P] dGTP, 150μg/ml of oligo(dT_{12-18}), 300μg/ml mRNA, 3000U. reverse transcriptase/ml at 43°C for 30min. cDNA synthesis was initiated by the addition of reverse transcriptase and the reaction was stopped by adding EDTA to 20mM. 1μl of reaction mixture was counted with Scintillation Counter to calculate the total count. To the reaction mixture, 15μl of phenol and $CHCl_3$ was added; the solution was blended vigorously in a Vortex mixer and then centrifuged and again 30μl of $CHCl_3$ was added to the aqueous phase, vortexed and centrifuged. The aqueous phase about 20μl was passed through 1ml Sephadex G-50 column to separate synthesized cDNA from unreacted deoxynucleotide triphosphates. The synthesized cDNA solution (300μl) was collected from the Sephadex G-50 column and 30μl were counted to calculate the percentage of the incorporation and the amount of synthesized first strand cDNA. Then ethanol and NH_4Ac were added to the solution as before and left at -20°C overnight to precipitate cDNA. After centrifugation, the pellet was washed with 75% ethanol first and then desiccated. cDNA was dissolved in 10μl of pH 8.0 TE buffer (10mM Tris-HCl, 1mM EDTA).

2). Synthesis of the second strand cDNA

The second strand cDNA was carried out sequentially 1h. at 12°C and 1h. at 22°C in 100μl system containing 50mM Tris·HCl pH 8.3 buffer, 0.4mM of dNTPs, 30μCi of [α-^{32}P] dGTP, 1μl of *E.coli* RNAseH (30U./μl), 5.6μl of DNA polymerase (280U./ml), and about 35ng of

synthesized first strand cDNA. EDTA was then added to 20mM to stop the reaction. The product was extracted with phenol and $CHCl_3$ mixture and then $CHCl_3$ and at last the aqueous phase was precipitated with ethanol and NH_4Ac same as described above. The pellet was dissolved in 10μl of pH 8.0 TE buffer. 1μl of the synthesized cDNA was counted to calculate the efficiency of the synthesis. The size of the first and second cDNA synthesized was checked by alkaline agarose gel electrophoresis.

7. Preparation of cDNA library

1). C-tailed ds-cDNA (Okayama 1982)

A. Trial tailing reaction from pUC8 cut by *Sma* I:

The number of tails to be tailed depends on the reaction time. If 10 C-tails were required for 0.1 pmol of *Sma* I cut pUC8 per 3' end, 50pM dCTP was used in the reaction, 2% dCTP would be tailed onto pUC8. A plot was then made by the percentage of incorporation against the reaction time. According to the percentage of incorporation expected, reaction time could be chosen from the plot.

0.1pmol of *Sma I* cut pUC8 was incubated with 10μl of 1M K-Cacodylate buffer pH 7.0, 5 μl of 10 mM of β-mercaptoethanol, 5 μl of 100 μM dNTPs, 1μl of 10 μCi [α-^{32}P]-dNTP, 2 μl of terminal transferase

(13u./μl), 5μl of 20 mM $MnCl_2$ at 30°C. At 5, 10, 15, 20, 30, 40, 50, 60min, samples were taken and TCA precipitated counts were counted; According to 2% incorporation of dCTP for 10 C tails, C-tailing reaction was carried out for 9 min.

2). C-tailing for ds-cDNA

The reaction was carried out the same as described above for 9 min except pUC8 was replaced by ds-cDNA. 1 μl of 10% SDS and 1 μl of 0.4M EDTA were added to stop the reaction. Then the mixture was extracted with phenol and $CHCl_3$ and the aqueous phase was chromatographed on Sephadex G-50 to separate the C-tailed dscDNA from unreacted deoxynucleotide triphosphates. The collected fraction was counted and precipitated with ethanol and NH_4Ac same as above at -20°C overnight. After centrifugation the pellet was dissolved in 20μl of TEN buffer (10mM Tris-HCl pH 7.5, 1mM EDTA, 150mM NaCl).

3). Annealing C-tailed ds-cDNA with G-tailed pUC9 (10 G-tailed)

1μl of C-tailed ds-cDNA (1ng) with 1μl of G-tailed pUC9 (10ng) supplied by BRL and 17 μl of TEN buffer in 20 μl system was incubated at 65°C for 5min first, then at 45°C for 2h. Finally the reaction was left in water bath which was switched off overnight and the mixture was stored at 4°C for next step.

At the same time self-annealing of G-tailed pUC9 was set up

under the same conditions as described above.

8. Transformation of *E.coli* with plasmid DNA

The following method was obtained from Douglas (1983). An overnight culture of the appropriate *E.coli* strain (in our experiment JM83 was used) was established in 10ml SO medium (Bacto tryptone 2%, yeast extract 0.5%, 10mM NaCl, 2.5mM KCl pH 7.5) This overnight culture (0.4ml) was used to inoculate a further 40ml SOB medium (40ml of SO medium containing 0.4ml of 1M $MgCl_2$ and 1M $MgSO_4$) and this culture grown at 37°C with shaking for 3-3$^1/_2$ h. until the optical density reached A_{550} =0.45 — 0.55. The culture was poured into two 50ml pre-cooled Oakridge tubes and cooled on ice for 15min. Then the cells were harvested at 4°C and 5,000 rpm for 12min in an MSE 8x50 rotor. The cells were gently resuspended in 13ml of ice-cold TFB(10mM K-MES pH6.2, 100mM KCl, 45mM $MnCl_2.4H_2O$, 10mM $CaCl_2.2H_2O$, 3mM $HACoCl_3$) and incubated on ice for 15min. The cells were harvested as above and gently resuspended in 3ml of ice-cold TFB.Then 112µl of ice-cold DMF was added into the cells and swirled gently and incubated on ice for 5 min. 112 µl of DTT (2.25M DTT in 40 mM KAc) was added to the cell solution in the same way and was incubated on ice for 10min followed by adding 112µl of ice-cold DMF into the cell solution in the same way and incubated on ice

for 5 min. The cells were now ready for transformation: 200 µl aliquots of the cells were dispensed into Falcon tubes. The DNA annealed to pUC9 vector 1µl (100ng) or pUC8 5µl (20ng/µl) as control was added to separate Falcon tubes containing cells and these were left on ice for 30min. The cells were then heat-shocked at 42°C for 90sec and then incubated on ice immediately for 1-2min. 800µl of SOC solution (SOB containing 8ml of 2M glucose) was added to the cells at room temperature and incubated at 37°C for 60min with shaking at 200rpm. 200µl aliquots of the above cell solution was placed LB containing ampicillin (100µg/ml) plate with 40µl (20mg/ml) of BCIG (X-Gal) and 40µl of IPTG. The plates were incubated at 37°C overnight. White potentially recombinant colonies were selected. Competent cells prepared in this way gave a transformation efficiency of about 1×10^7 colonies per µg of DNA.

9. Storage of cDNA library:

2000 large white colonies were picked individually into micro-titer plate wells containing 200 µl of LB(100µg/ml ampicillin), grown at 37°C overnight. 100µl of 50%glycerol were added to each well and mixed with sterilized mixer. The micro-titer plates were numbered and stored at -80°C.

10. Screening of cDNA library

The cDNA library was screened by probe either from nick translation of 3.6 Kb NADPH-GOGAT gene fragment or from labelled SP6 transcripts of NADPH-GOGAT gene:

1). Subcloning of *E.coli* NADPH-GOGAT gene fragment into pACYC184 and pGEM3

The recombinant plasmid pGOG2 (kindly supplied by Dr. Lightfoot) containing the GOGAT gene from 20Kb of the *E.coli* chromosome was digested with *Hin*d III. The 3.6 Kb GOGAT gene fragment located at the 3' end of the gene was isolated by elution from an agarose gel as described in section 3.1.4. 3.6Kb of GOGAT gene fragment was then ligated with *Hin*d III cut vector pACYC184 and pGEM3 respectively, then the two recombinant vectors were separately transformed into *E.coli* HB101.

A. Digestion of vectors

3μg (2μl) pACYC184 and 2μg (5.2μl) of pGEM3 were digested separately with 15U./1.5μl *Hin*d III at 37°C for 2h. 2μl (c.200ng) of each digestion sample was run on mini-agarose gel to check the digestion was complete or not (if complete, only one band appeared on the gel, without undigested and supercoiled DNA bands).

B. Dephosphorylation of vector DNA with calf intestinal phosphatase (CIP)

When the digestion was complete, the restricted plasmid DNA was dephosphorylated with calf intestinal phosphatase. Removal of terminal 5'-phosphate groups was achieved by adding 2µl of 10x CIP buffer (500 mM Tris-HCl pH 9, 10 mM spermidine, 10mM $MgCl_2$, 1mM $ZnCl_2$) to the digestion mixture along with 1µl (25u.) of CIP at 37°C for 1h.

C. Ligation of vector and 3.6Kb NADPH-GOGAT gene fragment

Ligation reactions were carried out in 10µl volumes containing 1µl (20ng) of phosphatased pACYC184 or pGEM3 vector and 2µl (100ng) of the 3.6Kb fragment. T4 DNA ligase 1µl (5u.) was used per ligation. 1µl of 10mM ATP, 0.5µl of 10mM Hexamine cobaltic chloride (HCC), and 1µl of fresh 10 X ligation buffer (0.5M Tris-HCl pH 7.5, 0.1M $MgCl_2$, 0.05M DTT) and 4.5µl ddH_2O were added and mixed. The reactions were incubated at 15°C overnight.

D. Transfomation of *E.coli* HB101 by ligated plasmid DNA and NADPH-GOGAT gene

2µl of the ligation mixture was used per transformation into *E.coli* HB101. The colonies containing 3.6Kb fragment from pACYC184 transformation were identified by double digestion of mini-prep plasmid

DNA with *Hin*dIII and *Bgl* II . The colonies containing 3.6Kb fragment from pGEM3 were identified by colony hybridization.

2). Preparation of probes

A. Nick translation probe:

3.6Kb fragment of NADPH-GOGAT gene was isolated from *Hin*dIII cut recombinant pACYC184 vector by agarose gel as described previously. The fragment was then labelled by nick translation using a modification of the method of Rigby *et al.* (1977). 4µl (150-200ng) of 3.6Kb fragment was incubated in a final volume of 20 µl reaction containing 2 µl of dNTPs (5mM of each), except dGTP, 2µl of 10 x nick translation buffer (0.5M Tris-HCl pH 7.8, 50mM $MgCl_2$, 100mM DTT), 5 µl of ^{32}P-dGTP (50µCi, 2mM), 1µl of freshly prepared DNase I (diluted to $25x10^5$ fold) 1.5µl DNA polymeraseI, 4.5 µl ddH_2O, at 15°C for 3h. Removal of the uninocorporated nucleotides was achieved by running the reaction mixture on a 1ml Sephadex G50 column. The elution fractions were checked by a monitor , the fractions from the first radioactivity peak were collected as the labelled probe.

B. SP6 transcription probe

A). Identification of colonies containing inserted DNA

Denaturation of the colonies

200 colonies from transformation by pGEM3 were picked and

transferred onto duplicated LB plates (containing ampicillin). The plates were kept overnight at 37°C in the incubator. Plates were then cooled on ice and nitrocellulose filter circle was placed on the surface and pressed gently by hand with glove. The filter was left onto the plate for 5min (NB: Make at least 2 reference marks on reference plates and nitrocellulose circles). The filter with colonies was denatured as following steps: 2 X 3MM papers were soaked in sandwich box lids(so that filter is wet but solution not running) with 0.5M pH 7.5 Tris-HCl buffer containing 1.5M NaCl. The nitrocellulose filter were transferred with forceps from the following one solution to the other at times indicated. Air bubbles between nitrocellulose and 3MM paper should be avoided.

1. 10% SDS	3min.
2. 0.5M NaOH	5min.
3. 1M Tris-Cl pH 7.5	5min.
4. 0.5M Tris-Cl pH 7.5, 1.5M NaCl	5min.

After colonies were denatured, nitrocellulose filter was put on a piece of clean 3MM paper. The filter was covered with another piece of 3MM paper. The solution on the filter was absorbed by rolling a rolling pin on the top of 3MM paper. Then 3MM paper was removed carefully and the filter was dried at room temperature for 30 min. Lastly, the filter was baked at 80°C for 2h. under vacuum.

Prehybridization

Nitrocellulose fiter was put into a polythene bag filled with

prehybridization solution (hybridization buffer without ssDNA and labelled probe) All air bubbles were removed and the filter was incubated in a 65°C waterbath with shaking for 1h.

Hybridization

Hybridization was carried out as described in Pall Biodyne Manual. Hybridization solution:

	Stock	5ml	10ml	20ml
5 X Denhardt's[1]	100X	0.25	0.5	1.0
5 X SSPE[2]	20X	1.25	2.5	5.0
0.2% SDS	10%	0.1	0.2	0.4
20µg/ml polyA,U or C	20mg/ml	5µl	10µl	20µl
100µg/ml salmon sperm DNA	10mg/ml	50µl	100µl	200µl
SDW		3.3	6.7	13.4

NB. 1. 100x Denhardt's solution is 2% (w/v) polyvinyl pyrrolidone-40, 2% (w/v) ficoll 400 and 2% (w/v) bovine serum albumin

2. 20 x SSPE: 3.6M NaCl, 0.2M Na_2HPO_4, 20mM EDTA pH to 7.7 with HCl

All ingredients were mixed except ssDNA and heated to 65°C.

Salmon sperm DNA with ^{32}P-probe was boiled for 10min to be denatured then was added to the mixture of ingredients.

Prehybridization solution was removed from the polythene bag and the hybridization solution was put in. After all air bubbles were removed, the bag was sealed and was sealed into another bag. Hybridization was

carried out carried out in a 65°C waterbath with shaking overnight.

The filter was washed with 6X SSC containing 0.1% SDS at 50°C for 0.5h for 3 times.The filter was baked at 80°C under vacuum and positive clones were identified according to the result of a X-film exposed onto the filter.

Checking the orientation of inserted DNA

The orientation which 3.6Kb fragment combined with pGEM3 was checked by running the digestion of the recombinant plasmid with *Pst* I on a mini-agarose gel (*Pst* I was chosen according to the restriction map available from Lightfoot, only one cut on the fragment by *Pst* I and in the multicloning site on pGEM3).

SP6 transcription

SP6 transcription was carried out following the method described by Melton *et al.*(1984). The pGEM3 plasmid containing 3.6Kb fragment in an orientation that gave the full length RNA transcripts was used for producing SP6 transcription probe. 5μg(20μl) pGEM3 containing insert was digested with 25U Sma I at 37°C for 2h. 1μl of the digestion was run on an agarose gel to check the digestion was complete or not. If complete, the reaction was phenol and $CHCl_3$ extracted to remove the remaining enzyme and proteins. After being precipitated with ethanol and washed with 75% ethanol, the pellet was desiccated and dissolved in 10μl of sterilized ddH_2O. SP6 transcription was carried out in a total volume of 20μl containing 2μl (1μg) of digested plasmid, 1μl of 200mM DTT, 1μl of

RNasin (placental ribonuclease inhibitor 15U./μl), 1μl of BSA (2mg/ml), 2μl of 5mM rATP, rCTP, rGTP, 1μl of 20mM rUTP, 2μl of ^{32}P-UTP (40μCi), 2μl of 10 X transcription buffer (400mM Tris-HCl pH7.5 containing 60mM $MgCl_2$, 20mM spermidine), 6.5μl sterilized water and the SP6 transcription was initiated by adding 0.5μl SP6 polymerase at 37°C and the reaction was carried out for 10 min and was stopped by adding 1μl of 0.4M EDTA. Then 29 μl of TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA) was added to make the volume of 50 μl. The mixture was extracted with phenol and $CHCl_3$. The aqueous phase was chromatographed by a 1ml Sephadex G50 column to separate the labelled transcription product from unincorporated rNTPs. The fractions from the first radioactivity peak were collected and transcription product was precipitated, washed, desiccated as before. The desiccated transcription product was dissolved in 20μl of TE buffer and was counted on Scintillation Counter. 2μl of transcription product ($5x10^5$ cpm) and labelled λ marker ($5x10^5$ cpm) were run on formamide gel as described previously to check the result of transcription and estimate the molecular weight of the product.

T4 Kinase ^{32}P labelling of λ marker

λDNA (*Hind* III cut) was phosphatased as described in section 3.1.10. The pellet (3μg) from phosphatased reaction was desiccated and dissolved in 25 μl TE buffer. The labelling reaction was carried out in a total volume of 30μl containing 10μl (1μg) of phosphatased DNA, 3μl of 10 x kinase buffer (0.5M Tris-HCl pH 7.6, 0.1M $MgCl_2$, 1mM spermidine

HCl, 1mM EDTA, 2mM DTT added before use), 1μl (10units) of T4 polynucleotide Kinase, 4μl (10μCi) of [γ-^{32}P]ATP and 10μl of ddH_2O. The mixture was incubated at 37°C for 30 min, then 2.5μl of EDTA was added to stop the reaction. The reaction mixture was extracted with phenol and $CHCl_3$. The supernatant was chromotographed by a 1ml Sephadex G50 column to separate the labelled DNA from unreacted [γ-^{32}P]ATP. After counting cpm, the labelled DNA was concentrated by ethanol precipitation.

3). Screening of the cDNA library

The cDNA library was screened on nitrocellulose filters and Biodyne filters with two kinds of probe respectively. The procedures were the same as described above. However, the conditions and hybridization solution were different when SP6 transcription probe was used. Prehybridization was carried out in the solution containing 5x SSC, 40% formamide, 5x Denhardt's , 0.1% SDS, 20μl/mg polyU (boiled for 10 min in advance) at 30°C overnight. Hybridization was carried out in prehybridization solution including boiled ssDNA and probe ($1x10^6$ cpm) at 30°C for two days.

4). Identification of potential clones

Plasmids from 10 potential clones chosen according to the hybridization results were prepared by mini-preps. These plasmids were digested with *Hind* III and *Eco*RI to release the cDNA inserts and were run

on agarose gel. pGEM3 with 3.6 Kb fragment plasmid was digested with *Hin*dIII and was also run on the gel as control. The gel was Southern blotted onto nitrocellulose filter and then hybridized with SP6 transcription probe.

A. Southern blotting

This was performed with a modified procedure of Southern (1975). Following visualization under ultra violiet, gel to be transferred onto hybridisation membrane was first treated with 500ml of depurination solution (0.1M HCl) in a plastic box with shaking for 20min. This process improves the transfer efficiency of larger DNA fragments. Then the gel was treated in the same way with 500ml of 1.5M NaCl, 0.5M NaOH to bring about denaturation of the DNA. After 20min the denaturing solution was replaced with another 500ml of fresh solution and the gel was treated for a further 20min. Then the liquid was replaced with 500ml of 1.5M NaCl, 0.5M Tris-HCl pH 7.4 to neutralize the gel. After 40min, the solution was replaced with the same volume of the same fresh solution for another 20min. The DNA was then transferred onto nitrocellulose or nylon membrane by capillary blotting. This was achieved by first placing the gel onto a platform overlain with Whatman 3MM paper dipping into a reservoir of 20xSSC. Areas on the platform surrounding the gel were covered with cling film to prevent the solution transferring directly from the reservoir to the membrane. The transfer membrane was pre-wet with 2x SSC and then placed on top of the gel. 2 layers of Whatman 3MM paper cut to the same

size as the membrane were placed over its exposed surface followed by paper tissues to a depth of 4cm. Finally the tissues were overlain with a glass plate on top of which was placed a weight of approximately 1Kg. Transfer of the DNA was allowed to proceed for 12-18hours. Following transfer of the DNA, membrane was briefly washed in 2 x SSC to remove any adhering agarose and allowed to dry for 30 min. The DNA was then fixed to the membrane by baking *in vacuo* for 2h at 80°C.

B. Hybridization of immobilised DNA to radiolabelled probe

The filter was hybridized with SP6 transcription probe to identify the plasmid containing the positive insert. Prehybridization, hybridization and washing condition were carried out exactely the same as cDNA library hybridized with SP6 transcription probe. The filter was backed at 80°C under vacuum and was radioautographed.

Chapter 3

Identification of NADH-GOGAT Two Isoenzymes from Root nodules of *Phaseolus vulgaris* L.

3.1. Introduction

GOGAT and GS work together in the pathway for the assimilation of symbiotically fixed nitrogen into organic form in root nodules of legumes supplying nitrogenous compounds for the plant growth. GS has been widely studied in a number of plant species. In *Phaseolus vulgaris*, GS has been found to be present as four different forms. In root nodules two isoenzymes of GS have been separated by ion-exchange chromatography. GS I is similar to the GS present in root, GS II is a nodule specific enzyme induced during nodulation (Cullimore *et al.*, 1983) and in parallel with the induction of nitrogenase. Besides nodule specific GS, several other nodule specific nitrogen assimilatory isoenzymes are induced during nodule development, such as uricase in soybean nodules (Bergmann *et al.* 1983), AAT in lupin (Reynolds and Farnden, 1979). It is worthwhile to determine whether a nodule specific GOGAT isoenzyme is induced during nodulation.

In higher plants two forms of GOGAT have been purified and characterised: Fd-dependent GOGAT and NADH-dependent GOGAT. Fd-GOGAT is mainly located in chloroplasts and has been purified from a number of species, whilst NADH-GOGAT is mainly located in root nodules of legumes and has not been studied widely because of the instability. The work on antibodies to Fd-GOGAT has shown that Fd-

GOGAT and NADH-GOGAT are two different enzyme proteins (Suzuki *et al.* 1984b). NADH-GOGAT in plant fraction of lupin nodules has been shown to be induced over a time course which paralleled the induction of nitrogenase and GS, thus suggesting that NADH-GOGAT is an important enzyme in assimilating ammonium in root nodules. However, Suzuki *et al.* (1984b)has reported that in soybean root nodules Fd-GOGAT is a major enzyme, its activity is twice as high as NADH-GOGAT activity. It is unclear which form of GOGAT in root nodules is more important in assimilating ammonium produced during dinitrogen fixation.

In this chapter, the activities of Fd-GOGAT and NADH-GOGAT in *Phaseolus vulgaris* root nodules have been compared and efforts on the separation of isoenzymes of NADH-GOGAT using different chromatographic techniques has been tried from *Phaseolus vulgaris* and from other species as well. Two isoenzymes of NADH-GOGAT have been identified and separated from root nodules of *Phaseolus vulgaris*.

3.2. Results

1. Stability of NADH-GOGAT

NADH-GOGAT from lupin root nodules has been reported to be

very unstable *in vitro* with a loss of activity of $2\%\cdot h^{-1}$ at 0°C (Robertson *et al.*1975b) and recently NADH-GOGAT from alfalfa was reported to be more unstable with the loss of activity of $3\%\cdot h^{-1}$ (Anderson *et al.* in press). Therefore, it is necessary to determine the factors required to maintain the stability of the enzyme so that accurate determinations of its activity in nodules can be made.

1) The effect of extraction buffers on NADH-GOGAT activity

The buffer used in the extraction of NADH-GOGAT was one of the factors affecting the enzyme stability (Table 3.1.). Hepes buffers and phosphate buffers were found to be able to stabilize the enzyme activity. However when nodules were extracted with Tris buffers, 70% of activity was lost at 0°C overnight. Similiar result was obtained by Boland and Benny (1977). They found that overnight dialysis against Tris·HCl buffer resulted in total loss of NADH-GOGAT activity.

Table 3.1. shows the differences in NADH-GOGAT activity when extracted from root nodules of *P.vulgaris* with different buffers.

Table 3.1. NADH-GOGAT activity extracted from root nodules of *P. vulgaris* with different buffers (at 0°C)

Extraction Buffer	NADH-GOGAT Activity (% of the assay within 5min after extraction)			
(50mM)	5min	1h	4h	24h
Hepes-KOH	100	100	93	50
Phosphate	100	99	92	46
Tris-HCl	100	94	88	20

The assay included 50mM pH 7.6 buffer, 1% β-mercaptoethanol, 1mM 2-oxoglutarate, 2.5mM L-glutamine, 0.16mM NADH.

2) The stability of NADH-GOGAT activity

The root nodules of *P.vulgaris* were extracted with pH7.6 Hepes buffer and desalted by Sephadex G-50 column into 50mM pH7.6 phosphate buffer. The activity of NADH-GOGAT was assayed at 25°C in 50mM pH 7.6 Hepes buffer containing 1% β-mercaptoethanol, 1mM 2-oxoglutarate, 2.5mM glutamine and 0.16mM NADH. The concentrations of the three substrates were all saturating for NADH-GOGAT. The stability of NADH-GOGAT activity was determined by incubating desalted extract of root nodules with different chemicals in assay mixture at room temperature and 0°C at different intervals (Table 3.2.). The result shows that enzyme was very unstable without stabilizers: at room temperature with total loss of the activity for 10 h. and 50% loss of the activity at 0°C. The enzyme from root nodules of *P.vulgaris* was observed to be most unstable compared to NADH-GOGAT from root nodules of lupin and alfalfa. The results showed that the presence of a sulfhydryl-protecting reagent such as β-mercaptoethanol (ME) or dithiothreitol (DTT) was essential to stabilize the enzyme activity. The inclusion of phenylmethylsulphonyl fluoride (PMSF), a protease inhibitor was important to prevent the enzyme from degradation. EDTA was also found to be useful in maintaining the enzyme activity. A combination of DTT or β-ME, PMSF and EDTA in Hepes or phosphate buffers resulted in a considerable effect on enzyme stability, with only loss of activity $0.5\%\cdot h^{-1}$.

Table 3.2. Stability of NADH-GOGAT in crude extracts of *P.vulgaris* root nodules

Additions	Activity of NADH-GOGAT (% of initial activity)		
	Room Temp.	0 °C	
	10h	10h	24h
None	0	50	10
10mM DTT	10	72	50
1%b-ME	8	66	48
1mM EDTA	12	60	44
1mM PMSF	18	80	60
1mM DTT+1mM EDTA +1mM PMSF	40	98	88
10mM KCl	4	48	40
10mM NH_4Cl	30	55	42
5mM Glutamine	8	52	44
5mM Glutamate	0	10	8
5mM 2-oxoglutarate	10	54	46

It was reported that NADH-GOGAT was also present in the bacteroids of root nodules (Robertson *et al.* 1975). To ensure that NADH-GOGAT activity in this study was extracted from plant cytosol, 0.5M sucrose was included in the extraction buffer to prevent the bacteroids membrane from breaking. A marker enzyme of bacteroids, β-hydroxybutyrate dehydrogenase could not be detected in the extract, indicating that there was no bacteroid contamination in the plant cell fraction and NADH-GOGAT activities determined in our work came from the plant fraction of root nodules.

From the results shown in Table 3.1. and Table 3.2., it can be seen that NADH-GOGAT activity could be stabilized with DTT or β-ME, EDTA and PMSF in either Hepes buffer or phosphate buffer. Therefore Hepes buffer was used to extract the enzyme from root nodules and K-phosphate buffer was used in the purification.

2. Comparison of NADH-GOGAT and Fd-GOGAT activities in *P.vulgaris* root nodules

It has been reported that Fd-GOGAT is also present in root nodules and in soybean root nodules activity of Fd-GOGAT is 2-fold higher than that of NADH-GOGAT (Suzuki *et al.* 1984b). No such a comparison has been reported between activities of Fd-GOGAT and NADH-GOGAT from root nodules of *P. vulgaris*. We have compared the activity of Fd-GOGAT and NADH-GOGAT from root nodules of both

P.vulgaris and soybean. Fd-GOGAT activity was routinely measured by the production of ^{14}C-labelled glutamate from ^{14}C-glutamine. NADH-GOGAT activity can be assayed either by measuring the oxidation of NADH at 340 nm or by measuring the production of ^{14}C-labelled glutamate. Therefore, a radiochemical method was adopted to compare the activities of two forms of GOGATs.

1) Checking the radiochemical methods

Before the comparison of the activities of two GOGATs was made, it was necessary to determine that the radiochemical method for both GOGATs was reliable. Nodules were extracted with pH7.6 Hepes buffer containing 0.5M sucrose, 10mM DTT, 1mM EDTA and 1mM PMSF. Fig 3.1.a. and b. show that the production of ^{14}C-labelled glutamate from both GOGATs was linear within 15min and increased as a function of the amount of extract of root nodules. Production of glutamate was not increased later than 15min in the case of NADH-GOGAT, suggesting that NADH-GOGAT was not as stable as Fd-GOGAT. The assay of the activity of two GOGATs have to be carried out within 15min.

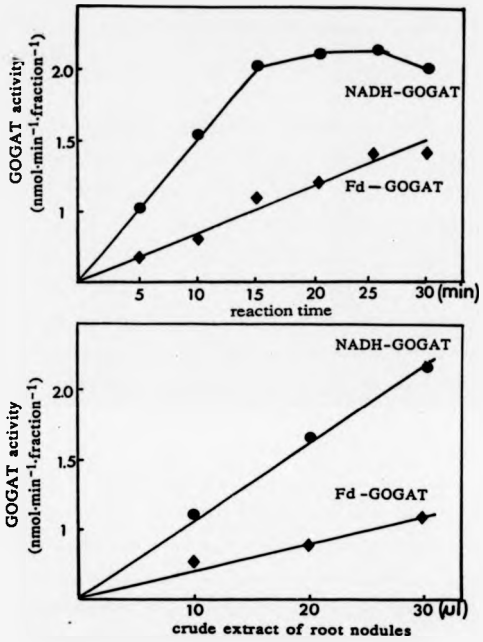


Fig.3.1. The production of ^{14}C-glutamate by the two GOGATs at different intervals and with different amount of extracts

2) A comparison of activities of NADH-GOGAT and Fd-GOGAT in root nodules of *P.vulgaris*

A comparison was made of NADH-dependent and Fd-dependent GOGAT activities in crude nodule extracts of *P.vulgaris* cv Tendergreen, using the radiochemical assay (Table 3.3.). Care was taken in the extraction procedures to minimise proteolysis and inactivation of the enzymes by including a protease inhibitor and activity stabilisers in the buffers and by preparing and assaying the extracts rapidly. Therefore the activities of both forms of GOGAT were relatively stable in the buffer used. The assay conditions were optimum for two enzymes. Both activities were found to be largely dependent on 2-oxoglutarate and reductant, and were abolished by azaserine an inhibitor of glutamine-amido transferases (Miflin and Lea 1980). The activity of NADH-GOGAT was found to be over three-fold higher than the Fd-GOGAT activity.

Measurements of NADH-GOGAT in these extracts using a spectrophotometric assay were 12.8nmol min^{-1} mg $protein^{-1}$ which was almost identical to a value 12.3nmol min^{-1} mg $protein^{-1}$ using the radiochemical method and therefore this simpler assay was used in all further experiments.

Table 3.3. Activities of NADH and ferredoxin–dependent GOGATin root nodules of *P. vulgaris*.

Assay conditions	GOGAT activity (nmol min^{-1} mg protein $^{-1}$)
Complete with NADH	12.3
— 2–oxoglutarate	0.2
+ azaserine (5mM)	0
Complete with ferredoxin	3.9
— 2–oxoglutarate	0.6
+ azaserine (5mM)	0
Complete — reductant	0.6

The activities were measured by the radiochemical assay as described in Methods.

3) A comparison of activities of NADH-GOGAT and Fd-GOGAT from soybean root nodules

The NADH-GOGAT activity and Fd-GOGAT activity in root nodules of soybean (*Glycine max* cv USDA Prize 110) were also compared by the radiochemical method (Table 3.4.). The root nodules were extracted with care to keep both NADH-GOGAT and Fd-GOGAT stable and activities were assayed immediately after extraction. The results showed that the substrate dependence and activities for NADH-GOGAT and Fd-GOGAT from soybean agreed quite closely with those found for *P.vulgaris* root nodules. Two GOGATs from soybean were dependent on 2-oxoglutarate and reductants, and were inhibited by azaserine. NADH-GOGAT activity was found about 3-fold higher than Fd-GOGAT.

Table 3.4.Activities of NADH and ferredoxin dependent GOGAT in root nodules of soybean (*Glycine max* cv USDA Prize 110)

Assay conditions	GOGAT activity (nmol min^{-1} mg protein $^{-1}$)
Complete with NADH	11.5
— 2-oxoglutarate	0.2
+ azaserine (5mM)	0
Complete with ferredoxin	3.4
— 2-oxoglutarate	0.3
+ azaserine (5mM)	0
Complete — reductant	0.4

The activities were measured by the radiochemical assay.

3. Identification of two isoenzymes of NADH-GOGAT in the plant fraction of root nodules of *P.vulgaris*

A crude extract of the plant fraction of *P.vulgaris* root nodules was prepared in 50mM Hepes pH 7.6 buffer containing 10mM DTT, 1mM EDTA, 1mM PMSF to stabilize the enzyme and 0.5M sucrose to keep bacteroid membranes intact. The extract was desalted by a 5ml Sephadex G-50 column. NADH-GOGAT activity was assayed and then the desalted extract was run on an HPLC ion-exchange column and the eluted fractions were assayed for NADH-GOGAT activity (Fig.3.2a.). Two peaks of activity eluted from the column at [KCl] of about 0.35 and 0.39M. The two peak fractions were then diluted about 10-fold to reduce the [KCl] to less than 40mM and were then rerun separately on the HPLC column (Fig.3.2b and c): each activity peak eluted at its original position on the [KCl] gradient. The two separated activity peaks were assayed for ferredoxin-dependent GOGAT activity and neither showed any activity with this reductant. We propose that these two activity peaks represent two isoenzymes of NADH-GOGAT which we have called NADH-GOGAT I and NADH-GOGAT II respectively.

To determine if there is any other NADH-GOGAT isoenzyme, with the same charge as either of the two separated isoenzymes but with a different molecular weight, the two nodule NADH-GOGAT activity peaks, were then run separately on an HPLC gel-filtration column. The fractions

were assayed for NADH-GOGAT activity and both NADH-GOGAT I and NADH-GOGAT II were reproducibly found to elute at a single activity peak and at slightly different positions (Fig.3.3b and c). This result suggested that only two NADH-GOGAT isoenzymes were present in root nodules of *Phaseolus vulgaris* and they have about the same molecular weight.

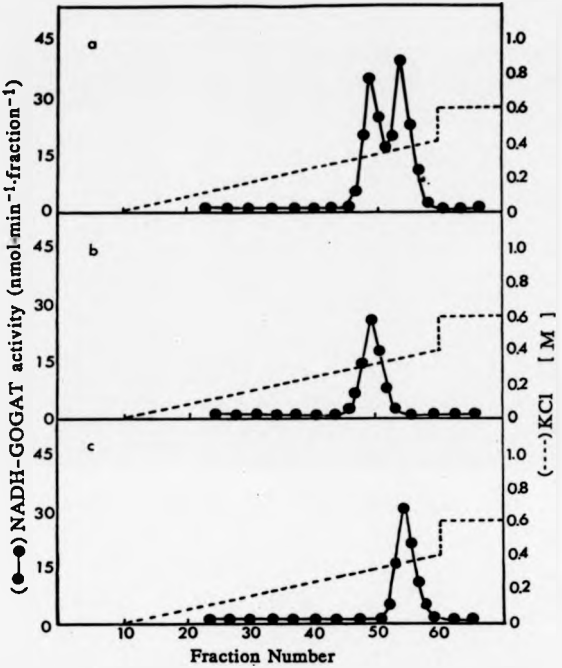


Fig.3.2. Elution profile of NADH-GOGAT activity by HPLC ion-exchange chromatography of

a. crude extract of root nodules from *P.vulgaris*

b.NADH-GOGAT I from root nodules of *P.vulgaris* separated by HPLC ion-exchange column

c.NADH-GOGAT II from root nodules of P.vulgaris separated by HPLC ion-exchange column

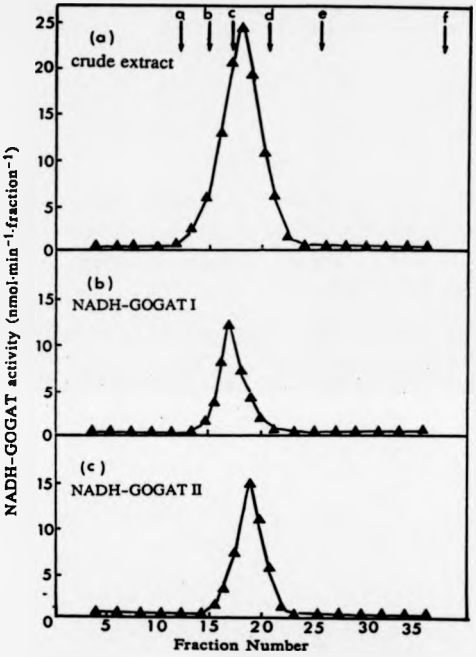


Fig.3.3. Elution profile of NADH-GOGAT activity from HPLC gel filtration column. Arrows from a—f represent the positions of molecular marker proteins (see Fig.3.4.) eluted from the column at the same condition.

4. Determination of molecular weight of two NADH-GOGAT isoenzymes

The molecular weight of the native two NADH-GOGAT isoenzymes were determined by HPLC gel-filtration chromatography (Fig 3.3.).

The crude nodule extract was also run on the HPLC gel-filtration column and the fractions were assayed for NADH-GOGAT activity (Fig. 3.3a.). Only a single peak of activity eluted from the column and in comparison to protein markers of known M_rs, the native M_r of NADH-GOGAT in crude nodule extracts appears to be about 200,000 (Fig 3.4).The native molecular weights of two nodule NADH-GOGAT isoenzymes corresponded to M_rs of about 210,000 and 200,000 for NADH-GOGAT I and NADH-GOGAT II respectively (Fig.3. 3b and c.).

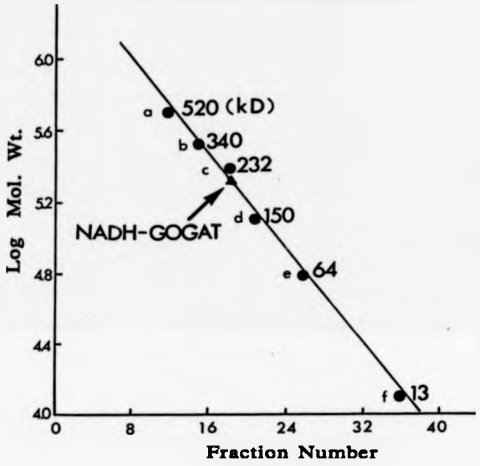


Fig.3.4. The measurement of molecular weight of NADH-GOGAT from crude nodule extract by HPLC gel filtration column compared to the known molecular weight marker enzymes: (a) β-galactosidase (M_r 520,000), (b) glutamate dehydrogenase (M_r 340,000), (c) catalase (M_r 232,000), (d) alcohol dehydrogenase (M_r 150,000) (e)haemoglobin (M_r 64,000), (f). cytochrome c (M_r 13,000).

5. NADH-GOGAT activities from other species

In *P. vulgaris* root nodules two isoenzymes of NADH-GOGAT were present. To check whether there exists isoenzymes with different charges in nodules of other legume species, the crude nodule extracts from soybean (*Glycine max* cv Peking 110 and USDA Prize 110), alfalfa (*Medicago sativa* L) and pea (*Pisum sativum* L.) were also run on the HPLC ion-exchange column in the same way respectively (Fig.3.5a.b.c.d). The eluted fractions were assayed for NADH-GOGAT activity. However, only one peak of NADH-GOGAT activity eluted from the column at slightly different [KCl] for all these species. The activity peak eluted from *Glycine max* cv USDA Peking 110 appears broader than others, it might contain two activity peaks with similar charges.

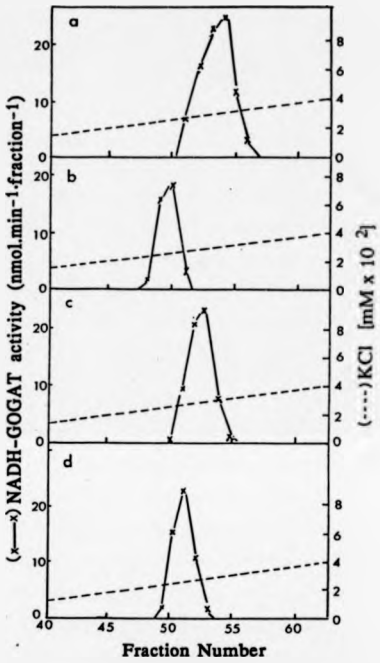


Fig.3.5. Elution profiles of NADH-GOGAT activity from root nodules of other species from HPLC ion-exchange column.

A.*Glycine max* cv Peking 110, B.*Glycine max* cv USDA Prize 110, C. alfalfa (*Medicago sativa* L), D.pea (*Pisum sativum* L.)

3.3. Discussion

NADH-GOGAT from *Phaseolus vulgaris* has been shown to be most unstable compared to the enzyme from root nodules of lupin and alfalfa. The activity of NADH-GOGAT could be stabilized by adding DTT or β-ME, EDTA and PMSF. The enzyme was found to be very unstable at room temperature and low (freezing) temperature. It was reported by Anderson *et al.*(1988) that NADH-GOGAT from alfalfa root nodules was stable when stored at -80°C in 50% glycerol, 100mM K^+-phosphate and 3mM DTT.

The activity of NADH-GOGAT is about 3-fold higher than that of Fd-GOGAT in *Phaseolus vulgaris* root nodules as measured by the radiochemical assay. This suggests that in root nodules of *P.vulgaris* NADH-GOGAT is the major enzyme working in conjunction with GS to assimilate ammonia produced by dinitrogen fixation. We also found that in soybean root nodules grown under our conditions NADH-GOGAT activity was about 3-fold higher than that of Fd-GOGAT (Table 3.4.). This observation is in contrast to the work on soybean nodules where the Fd-GOGAT activity has been shown to be twice as high as the NADH-GOGAT activity (Suzuki *et al.*1984b).The inconsistent result is probably due to the instability of NADH-GOGAT under the assay conditions and the extraction buffer used. It has been reported that Tris:HCl buffer decreased

the activity of NADH-GOGAT (Boland and Benny, 1977) and this has been confirmed by our experiment. We noticed that in the work on soybean root nodules Tris·HCl buffer was used. Therefore the relationship between NADH-GOGAT and Fd-GOGAT in root nodules and how they are regulated in assimilating NH_3 produced during dinitrogen fixation needs to be further determined.

The two NADH-GOGAT peaks could be reproducibly separated from crude nodule extracts by ion-exchange chromatography and each peak reran in its original position on the column (Fig.3.2.). It suggests that the two activity peaks are two separate enzymes. Separated two NADH-GOGAT activity peaks were run on HPLC gel-filtration chromatography, only a single activity peak was eluted for each at about the same position. It could be concluded from all the above results that two isoenzymes of NADH-GOGAT were present in the plant fraction of root nodules of *P. vulgaris* and could be separated by HPLC chromatography. The molecular weights of the two NADH-GOGAT isoenzymes were determined to be about 200,000 by HPLC gel-filtration chromatography. This value is the same as the M_r of alfalfa NADH-GOGAT (Anderson *et al.*, in press) and similar to lupin NADH-GOGAT (Boland and Benny, 1977).

Only one NADH-GOGAT activity peak was eluted from HPLC ion-exchange chromatography for other legume nodules. However, the activity peak of NADH-GOGAT from soybean Peking 110 was observed to be slightly broader than a single peak. It might consisted of two activity

peaks with similar charges. Whether there exist isoenzymes of NADH-GOGAT from other species remains to be determined. GS from root nodules of *Phaseolus vulgaris* was found to occur as isoenzymes but not in other species (Cullimore and Bennett, 1988).The occurrence of isoenzymes might be related to legume species.

Chapter4

Purification and Properties of NADH-GOGAT and the Production of Antibodies

4.1. Introduction

NADH-GOGAT has been purified and characterised from several species including lupin root nodules (Boland and Benny 1977), soybean cell culture (Chiu and Shargool 1979), etiolated stems of pea (Matoh *et al.* 1980), *P. vulgaris* (Awonaike 1980) and recently from alfalfa root nodules (Anderson et al., in press). Because of the difficulties in maintaining *in vitro* activity of NADH-GOGAT in extracts of root nodules, it brings a lot of difficulties in purifying the enzyme and producing the antibodies. This may explain why only a few studies have been done on NADH-GOGAT in root nodules in contrast to the work on GS and Fd-GOGAT. Only recently has an antibody raised against NADH-GOGAT from root nodules of alfalfa been reported (Anderson *et al.* in press). Considering the crucial role of GOGAT and the lack of knowledge of NADH-GOGAT in root nodules, it is important to purify the enzyme and to study the characteristics of the enzymes. To date NADH-GOGAT has been purified from *Phaseolus vulgaris* (Awonaike 1980) but before the discovery of the two isoenzymes.

In this chapter the purification of the two NADH-GOGAT isoenzymes is described. The kinetic and physical properties of the two isoenzymes have been examined. An antibody raised against purified NADH-GOGAT from SDS-polyacrylamide gel was produced and identified by Ouchterlony double diffusion and Western blot techniques.

4.2. Results

1.Purification of two isoenzymes of NADH-GOGAT

NADH-GOGAT is so unstable that 50% of the activity will be lost within several hours at 4°C if no stabilizers were included in the enzyme solution (Table 3.2.). Therefore the extraction buffer and all other buffers used in the purification must contain DTT or β-ME, EDTA and PMSF according to the result of stability experiment. Two isoenzymes of NADH-GOGAT were purified about 900-fold for NADH-GOGAT I and about 600-fold for NADH-GOGAT II and both proteins appeared homogeneous on SDS-polyacrylamide gel with silver staining. The procedures of purification are shown in Table 4.1. Ammonium sulphate precipitation after extraction from the nodule is not shown in this table but it was a necessary step to concentrate the soluble proteins of the nodules so as to run on Sephacryl-S300 column. Gel-filtration chromatography on Sephacryl S-300 column removed leghaemoglobin and other small proteins resulting in a 4.9-fold purification. The elution profile on the column of Sephacryl S-300 is presented in Fig 4.1. Although there exist two isoenzymes of NADH-GOGAT, their molecular weights are only slightly different, only one activity peak was eluted from the column. The Blue Sepharose column chromatography is shown in Fig 4.2. and resulted in a 51.4-fold increase in specific activity. This is an affinity chromatography

procedure; enzymes reacting with nucleotides such as NADH were absorbed on to the column. The NADH-GOGAT will bind onto the column in the presence of glutamate. The enzyme was then eluted with buffer containing 300mM NaCl and 50mM glutamine and 50 mM 2-oxoglutarate. In addition to the salt, the substrates glutamine and 2-oxoglutarate will change the conformation of the enzyme and hence cause disabsorption of the enzyme from the column. From the result of SDS-polyacrylamide gel, NADH-GOGAT was not however purified to homogeneity using this step. A relatively broad activity peak containing both isoenzymes was eluted. Not until chromatography on HPLC ion-exchange column, were the two isoenzymes separated completely (Fig.4.3.) and they were purified to apparent homogeneity after further chromatography on HPLC gel-filtration column (Fig 4.4.). The proteins at different stages during the purification procedures were run on SDS-polyacrylamide gel (Fig.4.5). Both proteins appeared to be homogeneous on SDS-polyacrylamide gels and revealed denatured M_rs of each of the two isoenzymes of about 200,000 (Track7 for NADH-GOGAT I, Track8 for NADH-GOGAT II). The total NADH-GOGAT activity recovered in the purification was about 8%, which was represented by about 2% and 6% for NADH-GOGAT I and NADH-GOGAT II respectively. The specific activities of NADH-GOGAT I and NADH-GOGAT II at the end of the purification procedures were 3.8 μmol min^{-1} mg $protein^{-1}$ and 8.1μmol min^{-1} mg $protein^{-1}$ respectively.

Table 4.1. The purification procedures of two NADH-GOGAT isoenzymes from root nodules of *P.vulgaris*

Purification step	Total activity ($\mu mol \cdot min^{-1}$)	Total protein (mg)	Specific activity ($\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$)	Purification* (fold)	Recovery (%)
Crude extract	21.50	1,248	0.017	1*	100
Sephacryl S-300 column	12.33	144	0.084	4.9	57.3
Blue Sepharose column	10.58	11.8	0.87	51.4	49.2
HPLC ion-exchange column					
NADH-GOGAT I	1.37	1.48	0.92	216	6.3
NADH-GOGAT II	3.36	1.94	1.73	136	15.6
HPLC gel filtration column					
NADH-GOGAT I	0.47	0.13	3.77	887	2.1
NADH-GOGAT II	1.26	0.16	8.05	631	5.8

* The fold purification of each isoenzyme was based on a determined activity ratio of NADH-GOGAT I : NADH-GOGAT II of 1 : 3 in crude nodule extracts.

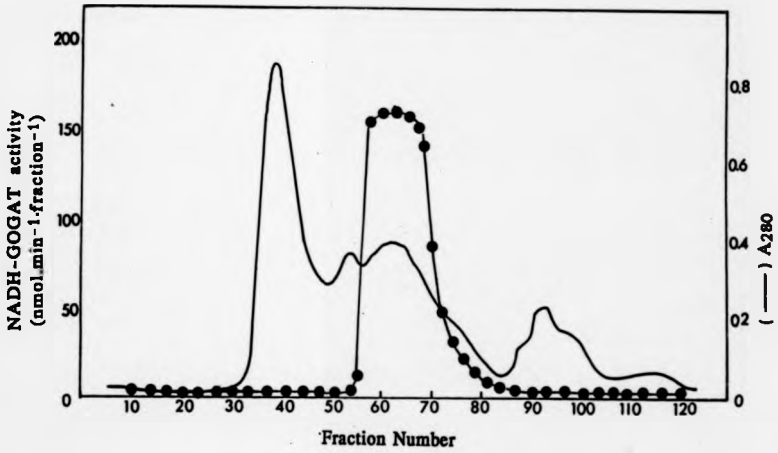


Fig.4.1. Elution profile of NADH-GOGAT activity from Sephacryl S-300 gel filtration column. •——• NADH-GOGAT activity

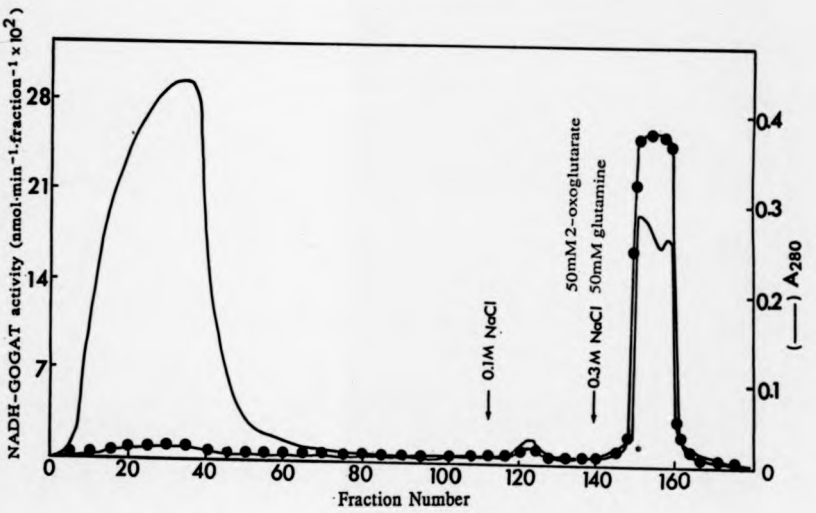


Fig.4.2. Elution profile of NADH-GOGAT activity from Blue-Sepharose column

•——• NADH-GOGAT activity

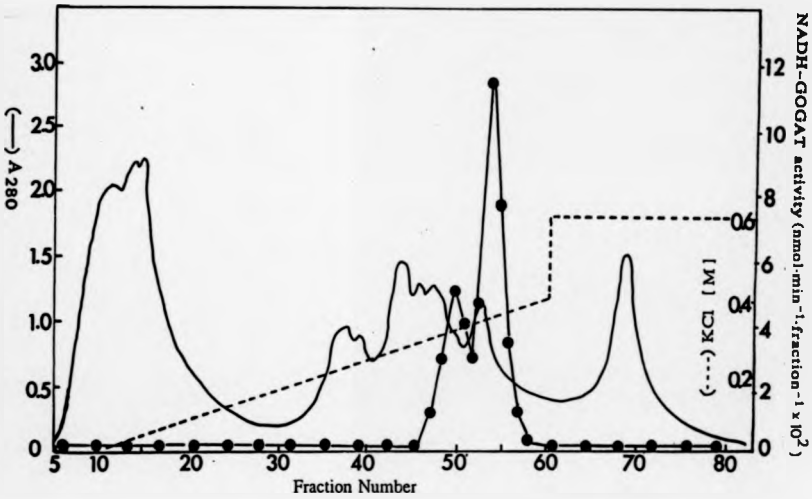


Fig.4.3. Elution profile of NADH-GOGAT activity from HPLC ion-exchange column

•——• NADH-GOGAT activity

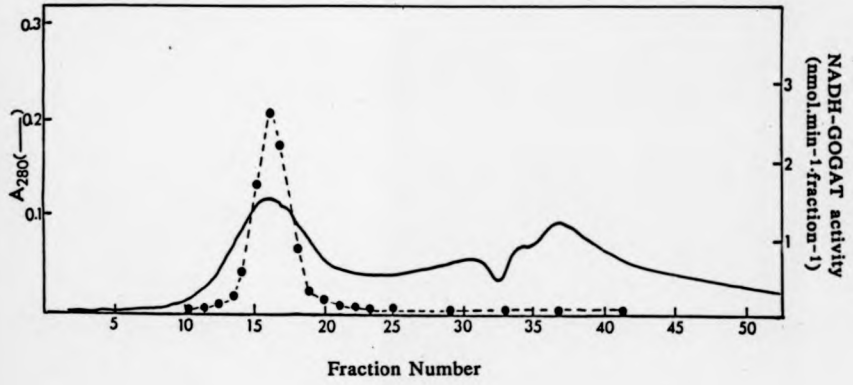


Fig.4.4. Elution profile of NADH-GOGAT I activity from HPLC gel filtration column
●----● NADH-GOGAT activity

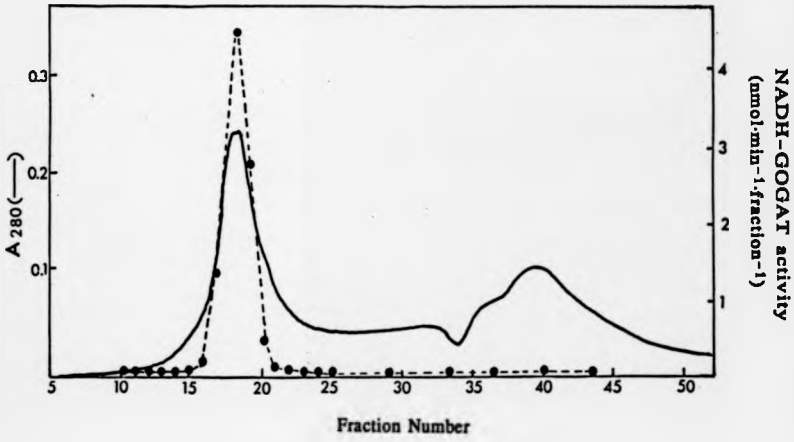


Fig.4.5. Elution profile of NADH-GOGAT II from HPLC gel-filtration column.
•----• NADH-GOGAT activity

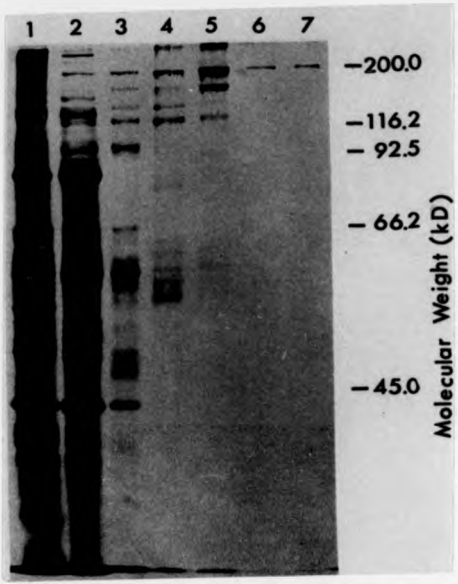


Fig 4.6. SDS-polyacrylamide gel of proteins from different purification steps. 15%SDS-polyacrylamide gel was run overnight at 10mA, the gel was silver stained. The proteins of each track from 1. crude extract (300μg) 2. Sephacryl S-300 column (200μg) 3.Blue Sepharose (10μg), 4. HPLC-ionexchange column for NADH-GOGAT I (5μg) 5. HPLC ion-exchange column for NADH-GOGAT II (5μg), 6. HPLC gel filtration column for NADH-GOGATI (0.5μg), 7. HPLC gel filtration column for NADH-GOGAT II (0.5μg). 8.The molecular markers: myosin (M_r 200,000), β-galactosidase (M_r116,200), phosphorylase B (M_r 92,000), bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000).

2. Activity characteristics of the two NADH-GOGAT isoenzymes.

1). Substrate specificity of the two NADH-GOGAT isoenzymes

Two NADH-GOGAT isoenzymes were separated and purified by ammonium sulphate precipitation, Sephacryl S-300 chromatography and HPLC ion-exchange column. Partially purified two isoenzymes of NADH-GOGAT were then used to study the specificity of the two isoenzymes for their substrates (Table 4.2.). From the table it can be seen that both NADH-GOGAT I and NADH-GOGAT II activities were totally abolished by adding azaserine, an inhibitor of amido-N transferases, to the reaction mixtures. The activities of both isoenzymes were largely dependent on their substrates L-glutamine, 2-oxoglutarate and NADH. Neither L-asparagine nor NH_4Cl could substitute for glutamine in the assay and neither pyruvate nor oxaloacetate could substitute for 2-oxoglutarate. Very little activity was measurable with either isoenzyme when NADH was replaced by NADPH.

Table 4.2. Substrate specificity of the two NADH-GOGAT isoenzymes from root nodules of *P.vulgaris*

Assay conditions	NADH-GOGAT activity (% of complete assay)	
	NADH-GOGAT I	NADH-GOGAT II
Complete assay*	100	100
+ 0.4mM azaserine	0	0
- glutamine	7.0	1.8
- glutamine + 1mM asparagine	7.0	0
- glutamine + 1mM NH_4Cl	3.0	0
- 2-oxoglutarate	3.6	3.6
- 2-oxoglutarate + 0.8mM pyruvate	2.8	1.6
- 2-oxoglutarate + 0.8mM oxaloacetate	3.2	2.6
- NADH	0	0
- NADH + 160 µM NADPH	2.6	0.8

* Complete assay includes 50mM Hepes pH 7.5, 1% β-mercaptoethanol, 1mM 2-oxoglutarate, 2.5mM L-glutamine, 0.16mM NADH.

2) Kinetic properties of the two isoenzymes

A. The determination of apparent K_m

The apparent K_m for each substrate was determined for each isoenzyme using partially purified enzyme (see NB on page 155). In each case the concentration of two substrates was saturated and kept constant while the concentration of the third was varied and the rate of reaction for each concentration of the third measured. The constant concentrations of glutamine, 2-oxoglutarate and NADH were respectively 2.5mM, 1.0mM and 0.16mM. The rates of reaction (v) were measured in arbitrary units (a.u.) which represent nmoles NADH oxidized per min per 0.05ml partially purified enzyme. The apparent K_m for each substrate was then determined by linear regression analysis of Lineweaver and Burk double reciprocal data. The K_m for each substrate of two isoenzymes was assayed twice and there were some variations between the two experiments. The results showed here were from the second experiment. Each point in the plot was the mean value of two assays which showed less than 5% variation (Fig.4.7., Fig.4.8. and Fig.4.9.). The results in Table 4.3. show that NADH-GOGAT I had an apparent K_m of 770μM for glutamine, 22μM for 2-oxoglutarate and 14μM for NADH. NADH-GOGAT II had an apparent K_m of 240μM for glutamine, 87μM for 2-oxoglutarate and 5.2μM for NADH. Therefore, NADH-GOGAT II had higher affinities for L-glutamine and NADH, but a lower affinity for 2-oxoglutarate when compared to the corresponding affinities for these substrates of NADH-GOGAT I.

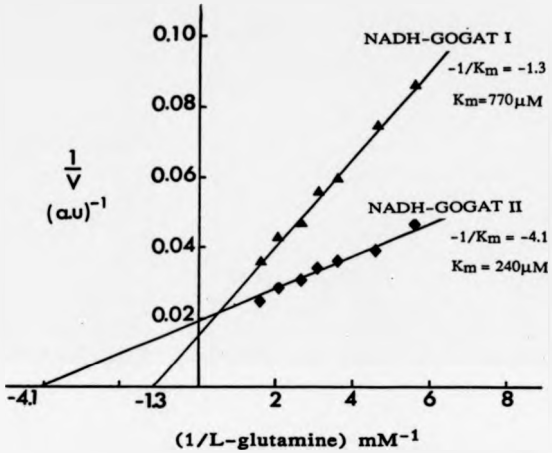


Fig.4.7. Determination of K_m for Glutamine of two NADH-GOGAT isoenzyme
The reaction was carried out at 30°C in the presence of 1.0 mM 2-oxoglutarate, 0.16mM NADH and 0.05ml of partially purified NADH-GOGAT isoenzyme. (Double reciprocal plot)

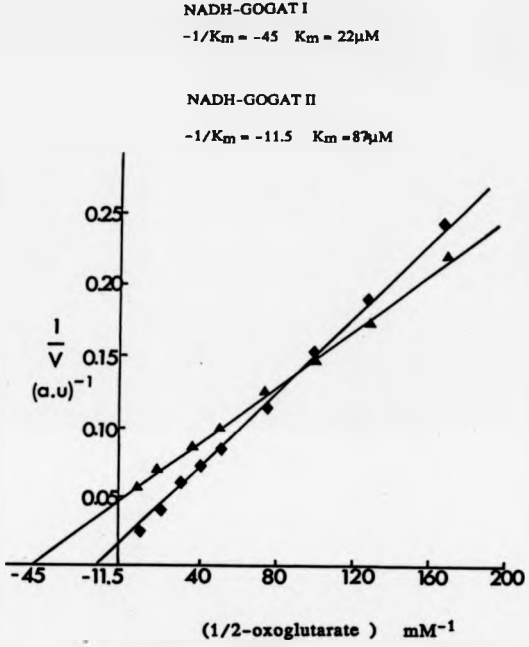


Fig.4.8. Determination of K_m for 2-oxoglutarate of NADH-GOGAT two isoenzyme
The reaction was carried out at 30°C in the presence of 2.5mM glutamine
0.16mM NADH and 0.05ml of partially purified NADH-GOGAT isoenzyme.
(Double reciprocal plot)

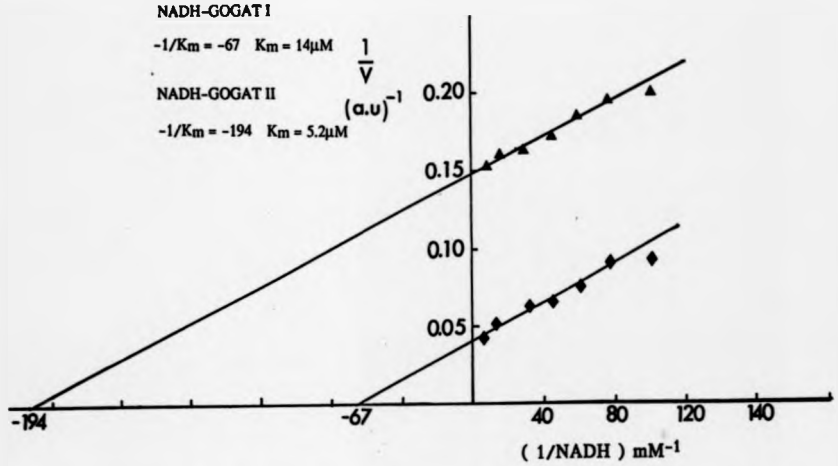


Fig.4.9. Determination of K_m for NADH of NADH-GOGAT two isoenzyme . The reaction was carried out at 30°C in the presence of 2.5mM glutamine 1mM 2-oxoglutarate and 0.05ml of partially purified NADH-GOGAT isoenzym. (Double reciprocal plot)

B. The determination of pH optima of two NADH-GOGATs

The pH optima (Fig.4.10) of the two isoenzymes were determined using partially purified enzyme at 25°C in 50mM Hepes buffers containing 1% β-mercaptoethanol, 1mM 2-oxoglutarate, 2.5mM L-glutamine, 0.16mM NADH. The concentrations of three substrates were saturated and constant while the buffer varied in pH. The rate of reaction was measured with different Hepes buffer. The pH optima of the two isoenzymes were different as shown in the pH profile (Fig.4.10.): NADH-GOGAT I had a broad pH optimum centering at pH 8.0 whereas NADH-GOGAT II had a much narrower pH optimum of 8.5.

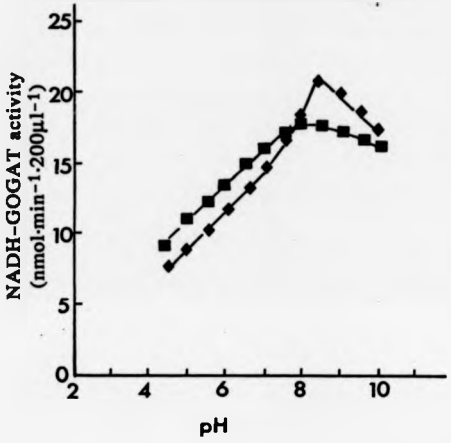


Fig.4.10. pH profiles of NADH-GOGAT two isoenzymes

■----■Activity of NADH-GOGAT I

◆----◆Activity of NADH-GOGAT II

C. The determination of temperature optima of two isoenzymes of NADH-GOGAT

The temperature optima for two isoenzymes were also determined using partially purified enzymes (Fig 4.11.). The assay was carried out in 50mM pH7.5 Hepes buffer containing 1% β-mercaptoethanol, 1mM 2-oxoglutarate, 2.5mM L-glutamine, 0.16mM NADH at 15°C, 20°C, 25°C, 30°C, 33°C,35°C and 38°C. It can be seen from Fig.4.11. that temperature optima for two NADH-GOGATs were about the same around 30°C.

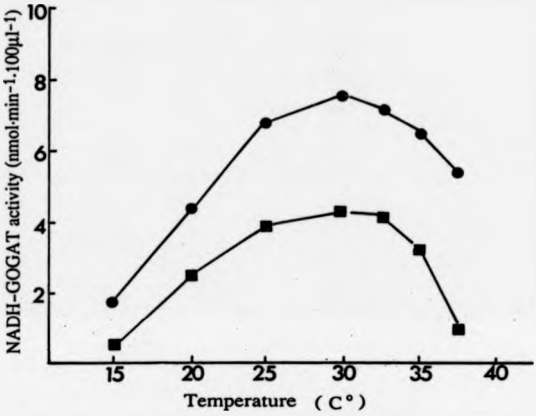


Fig.4.11. Temperature optima for NADH-GOGAT two isoenzymes

■ —— ■ Activity of NADH-GOGAT I

● —— ● Activity of NADH-GOGAT II

D. Kinetic properties of the two NADH-GOGAT isoenzymes from root nodules of *P.vulgaris* in relation to that from other species

Kinetic properties of the two isoenzymes of NADH-GOGAT were found to be slightly different except that temperature optima for two isoenzymes are about the same as shown in Table 4.3. However, all the values for kinetic properties were found to fall within the range for those from root nodules of other species (Table 4.4.).

Table 4.3. Kinetic properties of the two NADH-GOGAT Isoenzymes

Property	NADH-GOGAT I	NADH-GOGAT II
K_m for L-glutamine	770 μM	240 μM
K_m for 2-oxoglutarate	22 μM	87μM
K_m for NADH	14 μM	5.2 μM
pH optimum	8.0	8.5
Temperature optimum	30°C	30-32°C

The correlation coefficients obtained for the linear regression analyses of the K_ms were greater than 0.97.

Table 4.4. Kinetic properties of NADH-GOGAT from root nodules of *P.vulgaris* in relation to other sources

Source	pH optimum	Apparent Km values (μM) NADH	NADPH	Gln	2-Oxo	References
P. vulgaris (nodules) NADH-GOGAT I	8.0	14		770	22	
NADH-GOGAT II	8.5	5.2		240	87	
P.vulgaris (nodules)	7.6	7.1		222	18.5	Awonaike (1980)
G. max (cultured cells)	N.D.	9.0		630	64	Chiu & Shargool (1979)
G. max (cotyledons)	N.D.	8.3		1,100	630	Storey & Reporter (1978)
M. sativa (nodules)	7.5-8.5	4.2		466	33	Anderson *et al.* (1988)
L. angustifolius (nodules)	8.5	1.3		400	39	Boland and Benny (1977)
P.sativum (roots)		7.0		1,100	500	Miflin and Lea (1975)
P.sativum (cotyledons)	N.D.	13.3	27.7	1,430	960	Beevers & Storey (1976)
P.sativum etiolated shoots	N.D.	4.0		400	37	Matoh *et al.* (1980)
E.coli	7.6		7.7	250	17.8	Miller & Stadtman (1972)

N.D.= Not Determined

3). Stabilities of the activities of two NADH-GOGAT

The two partially purified NADH-GOGAT isoenzymes (see N.B. on page 155) were kept at room temperature, 0°C and -20°C separately. The activities of two NADH-GOGAT isoenzymes were assayed at 4h., 8h. and 24h. exactly the same as described in Methods. The results showed that both isoenzymes were unstable at room temperature, with 75-100% loss of activities for 8h. and neither were stable at low temperature. NADH-GOGAT I was more unstable than NADH-GOGAT II. NADH-GOGAT I lost total activity at room temperature for 8h and stored at -20°C overnight.

Table 4.5. Stabilities of the two partially purified isoenzymes of NADH- GOGAT from root nodules of *P.vulgaris*

		Activity of NADH-GOGAT (% of initial activity)	
		NADH-GOGAT I	NADH-GOGAT II
Room Temp	4h	35	68
	8h	10	25
	24h	0	0
0°C	4h	70	96
	8h	60	90
	24h	40	84
-20°C	4h	40	60
	8h	20	56
	24h	15	50

NB.

Two isoenzymes were partially purified by ammonium sulphate precipitation of the extract of root nodules, Sephacryl S-300 chromatography and HPLC ion-exchange chromatography.

4). Effects of various amino acids on the activities of the two NADH-GOGAT isoenzymes

The two partially purified NADH-GOGAT isoenzymes (see NB on page 155) were used to study the effects of amino acids on their activities. The enzymes were mixed with 20mM equal volume of amino acids first, 200µl of these solutions were then assayed for NADH-GOGAT activities exactly the same as described in Methods. Of the 19 amino acids tested, only asparagine, methionine and glutamine had no effects on the activities of both isoenzymes. Histidine, valine, tryptophan, threonine, lysine, leucine and serine inhibited both two isoenzymes by more than 50%. Phenylalanine inhibited NADH-GOGAT II by 30% but nearly had no effect on NADH-GOGAT I. Glutamate inhibited NADH-GOGAT I by 20% whilst NADH-GOGAT II only 10%.

Table 4.6. Effects of amino acids on the activities of two NADH-GOGAT Isoenzymes from root nodules of *P. vulgaris*

Amino acids	Inhibition (%) NADH-GOGAT I	NADH-GOGAT II
Ala	34	22
Asp	30	28
Asn	0	0
Arg	10	10
Cys	18	10
Glu	20	10
Gln	0	0
Gly	40	36
His	82	88
Hyp	30	26
Ile	10	14
Leu	70	62
Lys	58	60
Met	0	0
Phe	0	30
Pro	46	36
Tyr	76	80
Thr	74	78

The results are expressed as % inhibition of control (without amino acid). The activity of NADH-GOGAT was assayed in 50mM Hepes buffer, pH7.6, containing 1% β-ME, 2.5mM glutamine, 1mM 2-oxoglutarate and 0.16mM NADH in a final volume of 1ml. Activity of the control for NADH-GOGAT I was 14 nmoles NADH oxidized min^{-1} $100\mu l^{-1}$ and 18nmoles NADH oxidized min^{-1} $100\mu l^{-1}$ for NADH-GOGAT II. The final concentration of the added amino acids in the assay was 1.7mM.

5). Effects of salts on the activities of two NADH-GOGAT isoenzymes

The two partially purified NADH-GOGAT isoenzymes (see N.B. on page 155) were used to study the effects of different salts. Two isoenzymes were mixed with 20mM equal volume of different salts separately and 200µl of these solutions were then assayed for NADH-GOGAT activities exactly the same as described in Methods. Both NADH-GOGAT isoenzymes were inhibited by most divalent cations tested except Mn^{++}. Single valent cations tested either had no effects or increased the activities of two isoenzymes.

Table 4.7. Effects of salts on activities of two NADH-GOGATs

Salts	Inhibition (%) NADH-GOGAT I	NADH-GOGAT II
NaCl	4	2
LiCl	6	2
KCl	6	4
NH_4Cl	-18	-28
$CaCl_2$	80	72
$MgCl_2$	86	84
$MnCl_2$	10	8
$ZnCl_2$	100	96
$CoCl_2$	100	100

Complete assay includes 50mM Hepes pH 7.6, 1% β-mercaptoethanol, 1mM 2-oxoglutarate, 2.5mM L-glutamine, 0.16mM NADH. The activity was 14nmol NADH oxidized min^{-1} $100\mu l^{-1}$ for NADH-GOGAT I and 18nmol NADH oxidized min^{-1} $100\mu l^{-1}$ for NADH-GOGAT II. The final concentration of the added salts in the assay was 1.7mM.

3. Production and characterisation of antibody

In order to produce antibodies against the NADH-GOGAT isoenzymes, the two purified isoenzymes (50μg of each) from the last purification step (HPLC gel filtration chromatography) as described previously were injected into two rabbits on the back separately every two weeks. After the third injection the serum was taken and tested for antibodies by the Ouchterlony double-diffusion and Western blotting methods. No antibodies could be detected until the 9th injection. Considering the amount of purified enzyme was not enough, rats were replaced for producing the antibodies. Antibodies were not produced by rats when native purified enzyme was used. However, a rat produced antiserum against the purified M_r 200,000 dalton polypeptide isolated from SDS-polyacrylamide gel after the 4th injection.

1) Specificity of the antiserum

The specificity of the resulting antibody against purified NADH-GOGAT from SDS-polyacrylamide gel was checked initially by the Ouchterlony double-diffusion technique. This method is based on the migration of individual molecules through an agarose gel, their migration rates depending upon their initial concentration. When antibody interacts

with antigen a large macromolecular aggregate is formed which cannot diffuse further. This precipitate will prevent further diffusion of the particular antigen involved but not of another non-identical antigen. Fig. 4.11. shows the reaction of crude serum raised against the NADH-GOGAT at different dilutions (1, 1/2, 1/4, 1/8, 1/16) with root nodule extract in the central well. The titer of antibody is 1:8. The organ specificity of the antigen was checked by Ouchterlony double diffusion technique. Fig 4.12. shows the reaction of crude serum raised against the NADH-GOGAT (central well) with extracts from different organs and species. Extracts of root nodules from both *Phaseolus vulgaris* and soybean interacted with the antiserum and produced a precipitate line but the extracts from leaves, stems, cotyledons and roots of *P. vulgaris* did not interact with the antiserum.

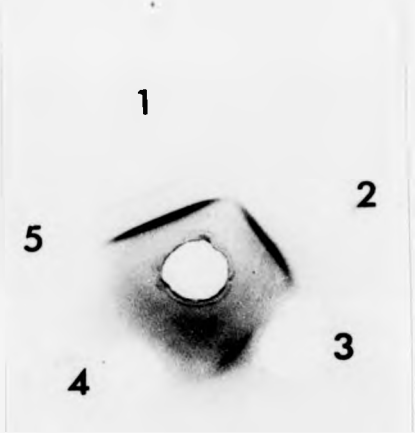


Fig.4.12. Titer of anti-NADH-GOGAT antiserum checked by Ouchterlony double diffusion using 10µl (0.1mg protein and 2nmoles·min^{-1}) of crude nodule extracts (central well) and anti-NADH-GOGAT antiserum at different dilutions (1) 1, (2) 1/2 , (3) 1/4, (4) 1/8, (5) 1/16.

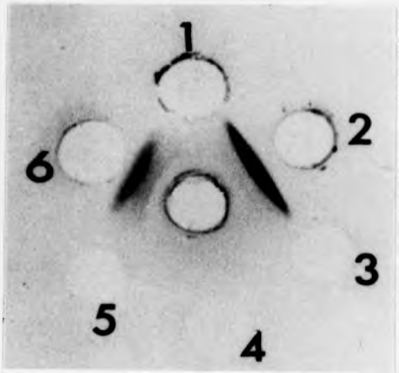


Fig.4.13. Organ-specificity of anti-NADH-GOGAT antiserum checked by Ouchterlony double diffusion using anti-NADH-GOGAT antiserum (central well) and crude extracts of (1) roots, (2) root nodules of *P.vulgaris*, (3) stems, (4) cotyledons, (5) leaves, (6) root nodules of soybean (*Glycine max* cv USDA prize 110).

2) Immunoprecipitation of NADH-GOGAT activity with the antiserum

However, when different amounts of antiserum were incubated with crude extract of root nodules followed by protein A, the recoveries of NADH-GOGAT activity in all the supernatants were about 90-95% (Fig.4.14a.). We thought that this might be due to rat IgG could not bind protein A properly. Rabbit anti-rat IgG was therefore used to bind protein A first followed by reacting with antiserum and the enzyme. After the incubation of extract of root nodules with antiserum, and rabbit anti-rat IgG with protein A, separately and two incubations were then mixed and incubated on ice for 90min, 90% of NADH-GOGAT activity was still present in the supernatant (Fig.4.14b.). Therefore the antiserum produced did not immunoprecipitate NADH-GOGAT from crude extract of root nodules.

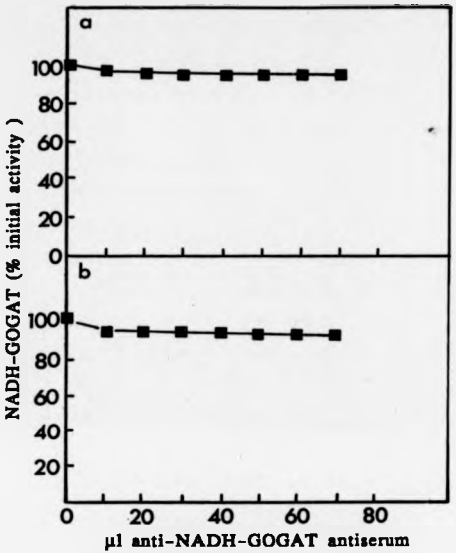


Fig.4.14. Immunoprecipitation of NADH-GOGAT activity by anti-NADH-GOGAT antiserum a. NADH-GOGAT from crude extracts of *P.vulgaris* root nodules (8nmoles.min^{-1}) was incubated with increasing volumes of anti-NADH-GOGAT antiserum and then incubated with ProteinA Sepharose b NADH-GOGAT from crude extracts of *P.vulgaris* root nodules (8nmoles.min-1) was incubated with antiserum,and ProteinA Sepharose was incubated with anti-rat-IgG separately, then two incubations were mixed and incubated. Each incubation was for 90 min. The solutions from a.and b.were centrifuged and NADH-GOGAT activities were assayed in the supernatents.

3) Identification of the antiserum by Western blotting

Characterisation of antiserum raised against NADH-GOGAT was also determined by "Western" blotting technique. The crude extract of NADH-GOGAT and the partially purified two isoenzymes were run on a 10% SDS-polyacrylamide gel and the proteins were blotted onto a nitrocellulose filter as described in Materials and Methods 13.3. The filters were incubated overnight with antiserum and subsequently incubated with [^{125}I]- protein A for 3h before exposure to X-ray film. From the result of autoradiography (Fig.4.15.), it could be seen that the antiserum reacted with M_r 200,000 protein bands from purified two NADH-GOGAT isoenzymes (track 2 and 3), crude extracts of soybean root nodules (track 1) and *P.vulgaris* root nodules (track4).

4) Organ specificity of NADH-GOGAT

Organ specificity of NADH-GOGAT was also checked by"Western" blotting. Fig.4.16. has shown that the antiserum reacted with M_r 200,000 protein bands from purified NADH-GOGAT two isoenzymes (track P1 and P2), crude extract of root nodules of *P.vulgaris* (track Cr). A smear around M_r 200,000 daltons could be seen from crude extract of roots but no discrete band and there was a hybridizing band from track of leaves which was M_r 140,000. There was no hybridizing band from crude extract of stems (track S).

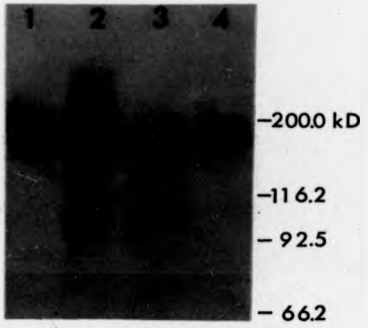


Fig 4.15. Western blotting result of checking anti-NADH-GOGAT antibody with samples of 1. crude extract of soybean root nodules (60μg) 2.purified NADH-GOGAT II(2μg), 3. purified NADH-GOGAT I (0.5μg), 4. crude nodule extract of *P.vulgaris* (60μg) and molecular markers (M): myosin (M_r 200,000), β-galactosidase (M_r 116,200), phosphorylase B (M_r 92,000), bovine serum albumin (M_r 66,200)

Fig 4.16. Western blotting result of checking NADH-GOGAT organ-specificity with samples of crude extract of L: leaves(100μg), S: stems(90μg), R: roots(100μg) Cr: nodules(100μg) and P2: purified NADH-GOGAT II(2μg) and P1: NADH -GOGAT I(0.5μg) of *P.vulgaris* and molecular markers (M): myosin (M_r 200,000), β-galactosidase (M_r 116,200), phosphorylase B (M_r 92,000), bovine serum albumin (M_r 66,200)

4.3.Discussion

Both two NADH-GOGAT isoenzymes were unstable at room temperature and low temperature; especially NADH-GOGAT I was extremely unstable compared to NADH-GOGAT II. It is very important that the purification has to be carried out as quickly as possible in cold room as well as including stabilizers in the buffers. However, HPLC chromatography has to be carried out at room temperature to prevent the column being damaged and the fractions were placed on the ice immediately after elution from the column. Most divalent cations tested could inhibit the activities of both two isoenzymes, thus EDTA was essential for stabilizing the activities of the two isoenzymes. The two NADH-GOGAT isoenzymes were purified to apparent homogeneity, at the end of purification checking by SDS-polyacrylamide gel. The specific activity of both NADH-GOGAT isoenzymes from *P.vulgaris* were low in this study compared to the specific activity of purified NADH-GOGAT from lupin nodules (Boland and Benny , 1977) and alfalfa nodules (Anderson *et al.*1988) but higher than that reported by Awonaike from the same species (1981). The differences in specific activities could be due to different species and the purity. The specific activity of NADH-GOGAT II was over two fold of that of NADH-GOGAT I. It could suggest that NADH-GOGAT II was a more efficient form in root nodules assimilating ammonium compared to NADH-GOGAT I. It should be pointed out that the pH optima were

measured after the two isoenzymes were discovered and purified. In all experiments in this work, the buffer used was pH 7.6, following the conditions of purification on NADH-GOGAT from lupin root nodules (Boland and Benny, 1977). Therefore the activities of both isoenzymes especially for NADH-GOGAT II were slightly underestimated in our work.

Using combined HPLC gel filtration and SDS-PAGE methods, the molecular weights of two NADH-GOGAT isoenzymes were determined to be about 200,000 monomeric polypeptides. This result was similar to the molecular weight determined for other species (Boland and Benny 1977, Awonaike 1981, Anderson et al. in press, Cullimore and Sims 1981). In all these work, NADH-GOGATs were reported to be monomeric proteins.

Kinetic properties for two isoenzymes from *P. vulgaris* were similar to those for NADH-GOGATs from other higher plants. NADH-GOGATs are specific for their substrates and only one or two NADH-GOGATs from higher plants can also use NADPH as cofactor (table 4.4.). NADH-GOGAT II had higher affinities for glutamine and NADH, but a lower affinity for 2-oxoglutarate when compared to the corresponding affinities for these substrates of NADH-GOGAT I. It can be seen that NADH-GOGATs in higher plants have apparent K_m values for NADH between 1.3 to 13μM, for glutamine from 240 to 1,100μM, for 2-oxoglutarate from 22 to 64μM (table 4.4.). All the enzymes from higher plants including *P.vulgaris*, have higher affinity for cofactor NADH and the enzyme affinity for glutamine is generally lower than that for 2-oxoglutarate. It could suggest that NADH-GOGATs may have a similar

kinetic mechanism in higher plants. Although the pH optima shown in Table 4.4. varied with different species, they ranged between 7 and 9. Two NADH-GOGAT isoenzymes had different pH optimum (Fig.4.10.), it suggests that they might have different locations.

Injection with native purified NADH-GOGAT to both rabbits and rats did not produce antiserum. It could be explained either the protein injected was not antigenic or native NADH-GOGAT was degraded soon after injected into the animals because of the instability. However, an antibody raised against purified native NADH-GOGAT from alfalfa was reported recently (Anderson *et al.* in press) and recognized specifically 200,000 bands from root nodules of several other legumes. The antiserum produced in this work was against purified NADH-GOGAT from SDS-polyacrylamide gel. Antiserum could crossreact with not only crude extracts of root nodules from *P.vulgaris* but also from soybean tested by Ouchterlony diffusion method. Crude extracts from other organs did not crossreact with antiserum, suggesting that antiserum specifically recognized proteins from root nodules. Furthermore, antiserum reacted with M_r of 200,000 polypeptides from crude extract of root nodules from *P.vulgaris* and soybean and purified two NADH-GOGAT when checking by Western blotting (Fig.4.14.), which indicated that the antiserum produced was anti-NADH-GOGAT antibody. The Western blotting methods was more sensitive than Ouchterlony double diffusion technique. The smear around M_r 200,000 in the track of roots could be very low amount of NADH-

GOGAT present in roots but was not detectable by Ouchterlony double diffusion. The hybridizing band about M_r 140,000 in the track of leaves might be a contaminating protein injected into rats which is also expressed in the leaves, but the size of this band agreed closely with M_r of Fd-GOGAT. Whether the antiserum raised against NADH-GOGAT from *P.vulgaris* could also recognize Fd-GOGAT or the size of the impurity was coincidentally the same as that of Fd-GOGAT was uncertain. However, no such band was seen when antiserum raised against NADH-GOGAT from alfalfa was used for Western blotting (Anderson *et al.* 1988). Furthermore, the antiserum raised against Fd-GOGAT from rice leaves could not recognize NADH-GOGAT (Suzuki *et al.* 1982). Several reports have shown that Fd-GOGAT and NADH-GOGAT are two different proteins (Suzuki and Gadal, 1984). The antiserum did not immunoprecipitate NADH-GOGAT from the crude extract of root nodules. This might be due to the antiserum raised against denatured protein from SDS-polyacrylamide gel failing to recognize the native enzyme. It might work if the antiserum was incubated with denatured crude extract rather than native enzyme followed by precipitation with protein A. The pellet should then be treated by triton and run on SDS-polyacrylamide gel; a M_r of 200,000 dalton band should be visible.

Chapter 5

Localization of the Two NADH-GOGAT Isoenzymes of *Phaseolus vulgaris* L.

5.1.Introduction

A lot of enzymes are involved in ammonia assimilatory pathways and considerable work has been done on the subcellular localizations of these enzymes. Since isolated bacteroids excrete NH_4^+ (Bergersen and Turner 1967), Robertson *et al.*(1975a) suggested that NH_4^+ is excreted to the host cell cytoplasm and is incorporated into glutamine and glutamate via the combined activity of GS and GOGAT. Several reports have shown that the majority of GS is located in the cytosol while NADH-GOGAT exists mainly in the plastids (Awonaike *et al.* 1981, Boland et al. 1982, Shelp and Atkins 1984, Shelp *et al.* 1983). Thus although $NH4^+$ is firstly incorporated into glutamine by the function of GS in the cytosol of plant cells of root nodules, the second reaction in the "Glutamate Synthase Cycle" (glutamine + 2-oxoglutarate via the activity of GOGAT to form glutamate) might be carried out in the plastids. A model for the pathway and subcellular localization of amide synthesis and ureide biogenesis in nodules of ureide-producing legumes has been proposed by Schubert (1986). In this model, the author proposed that the first reaction involved in "Glutamate Synthase Cycle" occurred in the cytoplasm of the nodule and glutamine produced was then transported into the plastids and plastid-located NADH-GOGAT catalyzed the transamidation of glutamine amido-nitrogen to the α-amino position of 2-oxoglutarate to form two moles of

glutamate. One mole of glutamate produced was transported back to the cytoplasm for the provision of substrate of GS. The other glutamate was used to synthesize other nitrogen-containing compounds. Since two NADH-GOGAT isoenzymes have been identified and separated in our experiments, it is important to determine the location of these two isoenzymes and hence the localization of the second reaction in the "Glutamate Synthase Cycle".

In this Chapter, sucrose gradient centrifugation following the method described by Boland *et al.* (1982) has been used to separate organelles and to study the location of NADH-GOGAT. A rapid isolation of plastids described by Boland and Schubert (1982) was adopted to determine the subcellular localisation of the two NADH-GOGAT isoenzymes.

5.2.Results

1. Separation of organelles from root nodules on sucrose gradients and determination of the subcellular localization of NADH-GOGAT activity

Freshly picked nodules were very gentle pressed and broken in a 0.5M sucrose containing buffer in order to isolate and maintain the integrity of the organelles. The extracted crude organelle solution was

loaded on continuous sucrose gradients as described in Methods. In addition to the plant cytosol portion with the characteristic leghaemoglobin, three distinct bands were visible after centrifugation. The separation of organelles was indicated by the marker enzymes, fumarase for mitochondria, triosephosphate isomerase or phosphoglycerate dehydrogenase for plastids and β-hydroxybutyrate dehydrogenase for bacteroids. These organelles were found at densities of 1.17, 1.21 and 1.23g/ml respectively. Fig 5.1 shows the distribution of marker enzymes for the three organelles and NADH-GOGAT of *P.vulgaris* root nodules following sucrose density gradient centrifugation. It can be seen from the figure that a lot of activity of triosephosphate isomerase (about 60%) from plastids was present in the cytosol fractions, this is higher than the value obtained by Awonaike (1981), suggesting much more breakage of plastids in this experiment. Fractions from the sucrose gradients were assayed for activity of NADH-GOGAT. The majority of NADH-GOGAT activity (65%) was found in the cytosol fractions. GOGAT activity decreased gradually as the sucrose density increased until the activity of NADH-GOGAT could not be detected in fractions of about 1.11g/ml of sucrose. GOGAT activity was not detectable in the fractions associated with mitochondria fractions between 1.15 and 1.18g/ml of sucrose. There was a definite activity peak associated with plastids (20%) in fractions between 1.19 and 1.23 g/ml of sucrose and 8% of total activity was observed in fractions between 1.22 and 1.25g/ml of sucrose, which was an overlap of plastids and bacteroids. β-hydroxybutyrate dehydrogenase activity was

only detectable after ultrasonic sonication of these latter fractions. However, NADH-GOGAT activity was not increased in these fraction after sonication; this suggested that NADH-GOGAT in these fractions was from the plastids. Yet, it was difficult to tell which isoenzyme of NADH-GOGAT was plastid located because the low recovery of NADH-GOGAT in the plastid fractions made it impossible to run this activity on the HPLC column to determine which isoenzyme was present.

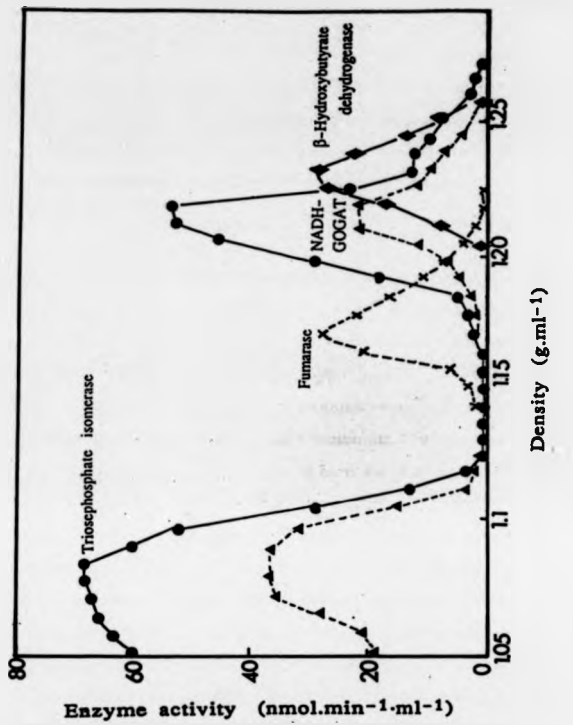


Fig.5.1. Separation of organelles from root nodules by sucrose density gradient centrifugation

The distribution of marker enzymes for mitochondria, plastids, and bacteroids and NADH-GOGAT of *P. vulgaris* root nodules. ●——● triosephosphate isomerase (for plastids, x 0.2), x---x fumarase (for mitochondria), ◆—◆ β-hydroxybutyrate dehydrogenase (for bacteroids) ▲---▲ NADH-GOGAT activity.

2. Isolation of plastid from root nodules and determination of the plastid location of the two NADH-GOGAT isoenzymes.

A method for rapid preparation of plastids reported by Boland and Schubert (1983b) was adopted to confirm the plastid location of NADH-GOGAT and to determine which isoenzyme was present in this fraction. Extreme care was taken to avoid plastids breakage during extraction as described in Methods. Nodule extract was spun down at 1,000 rpm for 4min to get rid of the debris and bacteroids. The suspension from the top of the centrifuge tube was loaded onto the discontinuous sucrose gradients. The plastids were separated by centrifuging for 30min at 145,000 x g. Fig.5.2. shows the tube after centrifugation. The major band is the plastids band. There was mitochondria contamination, according to the report by Boland and Schubert (1983b), below the pink cytosolic fraction after discontinuous sucrose gradient centrifugation, in the plastids band. The pink supernatent was taken off by pipette and the milklike plastid band was carefully taken through the tube with a syringe. The plastids fraction was prepared for running HPLC column by treating with triton X-100 to a final concentration of 0.1% and filtering through a 0.2μm filter.

Fig.5.3A and Fig.5.4B. show the activity profiles from HPLC ion-exchange column for GS and NADH-GOGAT from crude extracts of root noules of *P.vulgaris*. It can be seen from Fig.5.3.A. that the most GS

activity was eluted from the several first fractions which is the usual elution position of isoenzymes containing the γ-polypeptide of GS, located in the cytosol of root nodules. There was another higher GS activity peak eluted between 0.35 and 0.38M [KCl] which contained the mixture of γ and β-polypeptide of GS. The small GS activity peak eluted before this peak was mixture of γ, β, and α polypeptides of GS. The final small peak has been shown to be composed of the plastidic δ polypeptides (Cullimore and Bennett 1988). Two NADH-GOGAT activity isoenzymes were eluted from the crude nodule extract (Fig.5.4A). The activity of NADH-GOGAT II was twice that of NADH-GOGAT I in the crude extract of root nodules. Fig 5.3B. and Fig 5.4B. show the activity profiles from HPLC ion-exchange column for GS and two isoenzymes of NADH-GOGAT from the crude organelle extract of root nodules before loading on the sucrose gradients. In inner central cells of root nodules, GS activity was found to be mainly γ-polypeptide. There was another small but broad GS activity peak which is composed of mixtures of γ, β and α cytosolic subunits of GS. The final small peak was plastidic δ polypeptides. Fig. 5.4B shows that there were two activity peaks of NADH-GOGAT in the crude organelle extract. The ratio of activity of NADH-GOGAT II : NADH-GOGAT I was about 3:1, higher than the ratio for NADH-GOGAT II : NADH-GOGAT I (about 2:1) shown in Fig 5.3A. This suggested that NADH-GOGAT I was not recovered completely from the inner central cells of root nodules. Fig 5.3C and Fig 5.4C. show the activity profiles from HPLC ion-exchange column for GS and NADH-GOGAT from plastid fraction. GS activity was

detected in plastid fraction and eluted from HPLC ion-exchange column in the position corresponding to the isoenzyme composed of δ subunit which is plastid- located. The low activity of cytosolic GS was also detected in first several fraction from the column. Recovery of plastid GS in this fraction was about 49.5% whilst cytosolic GS was about 5.9% when compared to GS in the supernatent fraction. Both of two NADH-GOGAT isoenzymes were eluted from HPLC ion-exchange chromotography of plastid fraction but the ratio of activity of NADH-GOGAT II: NADH-GOGAT I (about 6:1) was much higher than that shown in Fig.5.4A. for normal extract of root nodules. The recoveries of plastid GS, plastid marker enzyme phosphoglycerate dehydrogenase and NADH-GOGAT I were about the same, around 50%, yet the recovery of NADH-GOGAT II in this fraction was about 75% (Table 5.1.). Fig 5.3D. and 5.4D. show the activity profiles for GS and NADH-GOGAT from HPLC ion-exchange chromatography of supernatant fraction of the gradients. The majority of activity of GS was eluted from the first several fractions and a very small GS activity peak was eluted from the column in the position of GS-δ. Both NADH-GOGAT isoenzyme activities were obtained from the supernatant, the ratio of NADH-GOGAT II : NADH-GOGAT I was about 1.5:1.

Fig.5.2. Rapid isolation of plastids by sucrose step gradient centrifugation. The sharp band below the pink cytosol fraction is plastid band, it was milk-white in colour not as pink as shown in this picture.

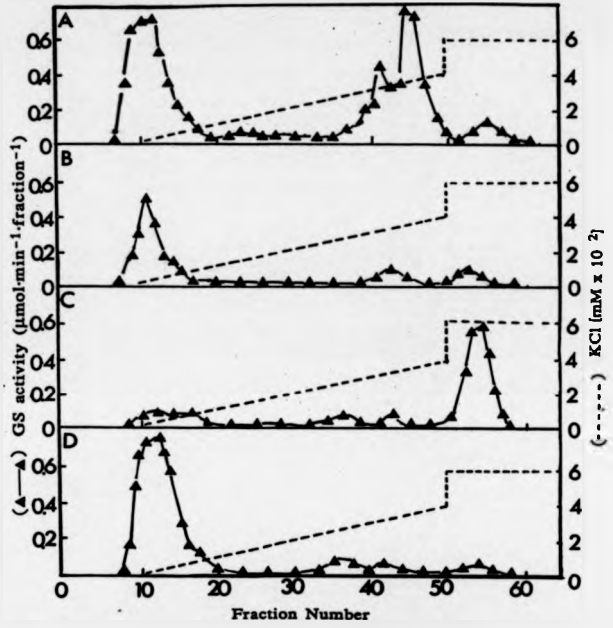


Fig.5.3. The elution profiles of GS activity from HPLC ion-exchange chromatography from: A. crude extract of root nodules, B. crude extract of organelles, C. plastids from sucrose step gradient centrifugation, D. supernatent from sucrose step gradient centrifugation

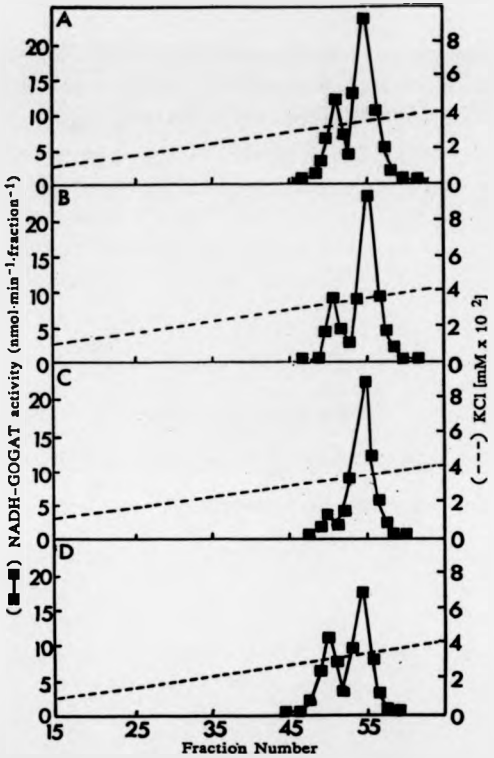


Fig.5.4. The elution profiles of NADH-GOGAT activity from HPLC ion-exchange chromatography from:

A. crude extract of root nodules, B. crude extract of organelles, C. plastids from sucrose step gradient centrifugation, D. supernatant from sucrose step gradient centrifugation

The recoveries of plastid GS, NADH-GOGAT two isoenzymes (after HPLC ion-exchange chromatography) and marker enzyme phosphoglycerate dehydrogenase activities from plastid fraction in comparison with the cytosol fraction from step gradient centrifugation of extract of inner cortical cells of root nodules is shown in Table 5.1. Plastid GS, marker enzyme PGDH and NADH-GOGAT I have about the same recovery and NADH-GOGAT II has much higher recovery when compared to the activities of these enzymes in the supernatent.

Table 5.1. Recovery of enzymes in plastid fraction

Enzyme	Percentage of total activity[1] (%)
NADH-GOGAT I	42
NADH-GOGAT II	75
GS (plastid)	50
PGDH[2]	45

NB.

1.total activity includes activities from supernatant and plastid

2 PGDH: phosphoglycerate dehydrogenase

3. Determination of the activity of the two NADH-GOGAT isoenzymes in the outer cortex

The activity of NADH-GOGAT I compared to the activity of NADH-GOGAT II in inner central cells was very low. This suggested that NADH-GOGAT I could be in the outercortex and it was discovered that NADH-GOGAT activity was present in this part of the nodules with specific activity of 7nmoles.min^{-1}.mg^{-1} protein. The outer cortex extract was prepared by pressing the nodule with a spatula in extraction buffer and washing the outercortex debris several times with ice-cold extraction buffer containing PMSF and DTT until white. This debris was then ground to completion with cold extraction buffer in a mortar and pestle. The extract of outercortex was run on the HPLC ion-exchange column and the fractions were assayed for GS and NADH-GOGAT activity. Fig. 5.5a and b. show the activity elution profiles for GS and NADH-GOGAT two isoenzymes from normal crude nodule extract and outer cortex extract. Comparing the ratio of two isoenzymes in nodule crude extract and outer cortex extract, NADH-GOGAT I was the major form in the outer cortex (67%), whilst NADH-GOGAT II was the major one in the crude extract of root nodules. It is surprizing that the activity of GS was eluted from the first several fractions correponding to nodule specific GS, γ-polypeptide. There was no other activity peak of GS detected from the eluted fractions. The ratio

of two isoenzymes of NADH-GOGAT in different extracts of root nodules was different (Table 5.2.), which could give hints for the locations of two NADH-GOGAT isoenzymes.

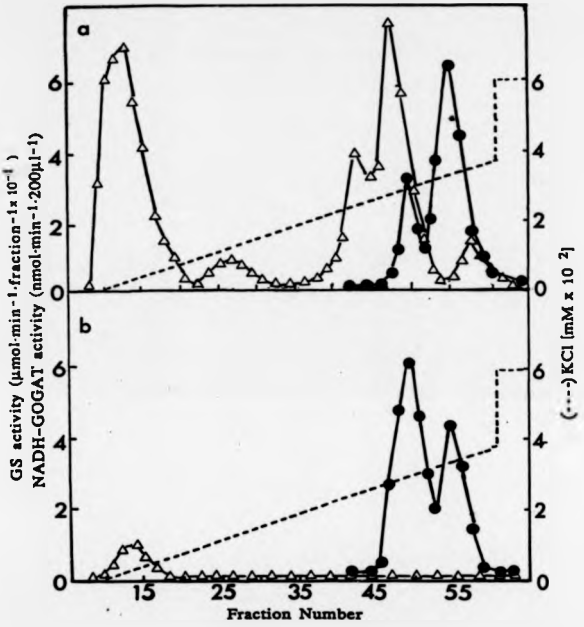


Fig.5.5. Activity profiles of HPLC ion-exchange chromatography for GS (Δ—Δ) and NADH-GOGAT (•——•) from a. crude extract of root nodules
b.outercortex of root nodules of *P.vulgaris*

Table 5.2. A comparison of the activity ratio of two isoenzymes of NADH-GOGAT in different extracts of root nodules of *P.vulgaris*

Extract	NADH-GOGAT I	:	NADH-GOGAT II
Crude extract of root nodules	1	:	2
Crude extract of organelles	1	:	3
Plastids	1	:	6
Supernatent	1	:	1.5
Outer cortex	1	:	0.5

4. Western blotting of GS from different organelle fractions

GS activity was obtained from plastids isolated and was eluted at the GS-δ subunit position from HPLC column. Plastid-located GS can also be detected by Western blotting with GS antibodies. Plastid GS subunits have a higher M_r than nodule cytosolic GS subunits on SDS-polyacrylamide gel besides it was eluted off later from HPLC ion-exchange column than other polypeptides of GS (Bennett, personal communication.). The crude nodule extract, crude organelle extract, outercortex extract, plastid fraction and the plastid GS fraction from HPLC ion-exchange column were Western blotted onto nitrocellulose filter and incubated with GS antiserum followed by detection with a peroxidase-linked system as described in Methods.

Fig.5.6. shows the result of Western blot of GS from different organelle fractions. GS from the HPLC ion-exchange chromatography of plastid fraction gave a single band which was in the position of δ polypeptide of GS (track 3, very faint) but no bands were apparent for the plastid fraction sample isolated from the sucrose gradients. It might be due to the degradation of plastid GS during storage of the crude plastids at -80°C for a week. GS from crude organelles extract and crude nodule extract gave two bands which were γ and β polypeptides of GS (track1 and 2). GS from the outer cortex gave a band mainly composed of γ polypeptide of GS (track 5).

Fig.5.6. Western blotting of GS from different organelles of root nodules of *P.vulgaris* GS from Track1: extract of root nodules,Track2: crude extract of organelles from root nodules ,Track3: plastids from HPLC ion-exchange column Track4: plastids from sucrose step gradient centrifugation Track5: extract of outer cortex of root nodules.

5.3. Discussion

The sucrose gradient centrifugation resulted in the separation of mitochondria, plastids and bacteroid fractions as indicated by marker enzymes (Fig 5.1.). NADH-GOGAT activity was found in in both the cytosol and plastid fractions. A plastid location of NADH-GOGAT from *Phaseolus vulgaris* was therefore confirmed by this study and has already been reported by Awonaike *et al.* from *P. vulgaris* (1981) and by Suzuki *et al.* from soybean root tissue (1984b). The distribution of NADH-GOGAT from this study was similar to that from soybean root nodules reported by Boland *et al.* (1982). The majority of NADH-GOGAT activity was recovered in the cytosol fractions along with the plastid marker enzyme triosephosphate isomerase, suggesting breakage of the plastids occurred during extraction of crude organelles from the nodules. However we could not exclude the possibility of some cytosolic location of NADH-GOGAT. The low activity of NADH-GOGAT in bacteroids fraction was due to the contamination of plastids, because the activity of NADH-GOGAT was not increased after sonication and centrifugation; this has also been reported by Awonaike *et al.* (1981).

Plastids isolated by rapid isolation method was satisfactory for identification of the localisation of the two NADH-GOGAT isoenzymes.

Although there was a contamination by mitochondria, it has been shown in Fig.5.1. that NADH-GOGAT activity was not detected in the mitochondria fraction and no evidence from other workers has suggested a mitochondria associated NADH-GOGAT. 49% of GS-δ, 45% of PGDH and only 6% of cytosolic GS activities were recovered from this fraction when compared with the activities of these enzymes from supernatent. This indicates that the fraction isolated from rapid sucrose gradient centrifugation did contain plastids and had only a low contamination of the cytosol. Both NADH-GOGAT isoenzyme activities were obtained in the plastid fraction which could suggest that both NADH-GOGAT isoenzymes are located in plastids of inner central cells of root nodules. From Table 5.2. by comparing the ratio of two isoenzymes of NADH-GOGAT in different extracts, it could suggest different cellular locations of the two isoenzymes. The ratio of NADH-GOGAT II : NADH-GOGAT I was higher in plastid fraction of inner central cells of nodules than in crude extract of nodules. This suggests that NADH-GOGAT I was not recovered completely and is perhaps present in outercortex cells of the nodules. The data obtained from determination of the activity of the two NADH-GOGAT isoenzymes in outercortex has confirmed this suggestion. The ratio of NADH-GOGAT II : NADH-GOGAT I was much higher in plastids of inner central cells of nodules, this suggests that NADH-GOGAT II is mainly located in plastids of inner central cells whilst only a small portion of NADH-GOGAT I is probably present in this part. The ratio of NADH-GOGAT II : NADH-GOGAT I was found to be lower in outercortex than in crude extract of

nodules which indicates that the activity of NADH-GOGAT in the outercortex is mainly from NADH-GOGAT I; low activity of NADH-GOGAT II might be due to the contamination of the inner central cells of the nodules.

The recoveries of NADH-GOGAT I (42%) in plastids was found to be close to that of two plastid marker enzymes GS-δ (49%) and PGDH (44.9%). Yet recovery of NADH-GOGAT II was much higher, about 75%. The differences in recoveries in plastids of the two NADH-GOGAT isoenzymes could be explained that NADH-GOGAT I is located in the same cell types as the two marker enzymes, whilst NADH-GOGAT II might be located in different cell types. However, the lower recoveries for GS-δ, PGDH and NADH-GOGAT I could also result from the underestimation of the activities of these three enzymes due to instability in addition to differential plastid breakage. The low percentage of NADH-GOGAT I (compared to NADH-GOGAT II) from inner central cells resulted in the identification of this isoenzyme from extract of outercortex. However, the result has shown that NADH-GOGAT I is also present in inner central cells of root nodules.

Western blotting of GS from outercortex of root nodule gave a band mainly nodule specific GS-γ (Fig.5.6). This was in agreement with the result of the activity profile of GS in outercortex from HPLC which also showed that low activity of GS was eluted in the position of GS-γ. Similarly, NADH-GOGAT II was also obtained in the extract of outercortex. Whether this reflects that GS-γ and NADH-GOGAT II are also

present in outercortex or that there is contamination by the inner central tissues remains to be determined. GS from crude organelles and crude extract of root nodules were the same, gave two bands, GS-γ and GS-β. There was no visible band in Track 4, which was the plastids fraction from sucrose gradients. It could be due to the instability of GS-δ during the storage as GS-δ has been found to be very unstable (Bennett, per commun.). The presence of GS-δ in fraction of plastids was confirmed by the result of Track 3, a very faint band was observed in the position of GS-δ polypeptide, slightly higher M_r than GS-γ and GS-β.

Chapter 6

Regulation of the Expression of Two NADH-GOGAT Isoenzymes of *Phaseolus vulgaris* L.

6.1 Introduction

A lot of evidence has shown that during nodulation a series of nodule specific ammonia assimilatory enzymes and the most abundant nodule specific protein leghaemoglobin are induced and increased over a time course similar to nitrogenase (Robertson *et al.*, 1975a, 1975b, Verma *et al.* 1981, Sengupta-Gopalan and Pitas 1986, Nguyen *et al.*, 1986) The expression of these nodule specific proteins might be under the control of the same regulatory system. Studies of nodule development in cowpea seedlings, cultured with their nodulated root systems exposed to atmospheres of 80%Ar: 20%O_2, indicated that while high levels of nitrogenase activity were expressed under these conditions the development of pathways of ammonia assimilation were severely depressed and the findings suggested a specific role of ammonia production or of some product of its assimilation in regulating the activity of the enzymes involved in ammonia assimilation (Atkins *et al.*, 1984). Since two NADH-GOGAT isoenzymes have been found to occur in root nodules and the localization and kinetic properties of the two isoenzymes have been observed to be different, the regulation of expression of these two isoenzymes during nodule development could also be different. The study in this Chapter is aimed at determining the regulatory factors affecting the activity of the two NADH-GOGAT isoenzymes during nodule development. The experiments involved

(1) to study the activity changes in the two NADH-GOGAT isoenzymes in nodules formed with wild type *Rhizobium* during nodule development to determine the relationship of the activity of the two isoenzymes.

(2) to study the activity changes in the two NADH-GOGAT isoenzymes in root nodules formed with Fix^- mutant *Rhizobium* CE 108, compared to wild type CE 3 in order to determine whether NADH-GOGAT two isoenzymes are synthesized when there is no NH_4^+ produced by nitrogen fixation.

(3) to study the activity changes in the two NADH-GOGAT isoenzymes in nodules formed under Ar, compared to that under N_2 to determine whether NADH-GOGAT two isoenzymes are synthesized in absense of N_2 fixation in nodules formed with normal *Rhizobium*.

(4) to study the activity changes in the two NADH-GOGAT isoenzymes when exogenous NH_4^+ supplied to (a) uninoculated root system, (b) nodules formed with Fix^- mutant *Rhizobium*, to determine whether exogenous NH_4^+ can cause the expression of NADH-GOGAT two isoenzymes.

(5) to study the organ-specificity of the two NADH-GOGAT isoenzymes in *P. vulgaris*.

6.2. Results

1. Changes in activity of the two NADH-GOGAT Isoenzymes During Nodule Development.

Five-day old *P. vulgaris* seedlings were inoculated with *R.leguminosarum* bv *phaseoli* (day 0) and nodulating roots or nodules were harvested at various times during development and assayed for nitrogenase and NADH-GOGAT activity (Fig.6.1.). Nitrogenase activity was first detectable on day 10 following inoculation and increased dramatically from day 10 to day 20. NADH-GOGAT activity was detectable even in the youngest samples but increased about 27-fold in specific activity up to day 18 over a time course similar to the increase in nitrogenase activity. The proportion of NADH-GOGAT activity attributable to NADH-GOGAT I and NADH-GOGAT II was determined by separating the two isoenzymes by ion-exchange chrmatography (Fig.6.2.). A 70 to 80% recovery of NADH-GOGAT activity was routinely obtained in these procedures. Uninoculated roots contained very low NADH-GOGAT activities (about 0.63 nmol min^{-1} mg $protein^{-1}$) and was comprised largely of NADH-GOGAT I. This isoenzyme was detectable in nodules throughout the development period and was the only isoenzyme present in the nodulating root systems at day 5. NADH-GOGAT II was

detectable at day 8 (at a time when nodules were clearly visible) and its activity increased in proportion throughout nodule development and at day 20 was about twice as active as NADH-GOGAT I. When the activities of each isoenzyme were expressed as specific activities (Fig.6.1.) it can be seen that NADH-GOGAT I activity increased about three-fold until about day 14 and then remained constant. However, a much greater increase in specific activity was observed for NADH-GOGAT II and this isoenzyme accounted largely for the total increase in NADH-GOGAT activity during nodule development.

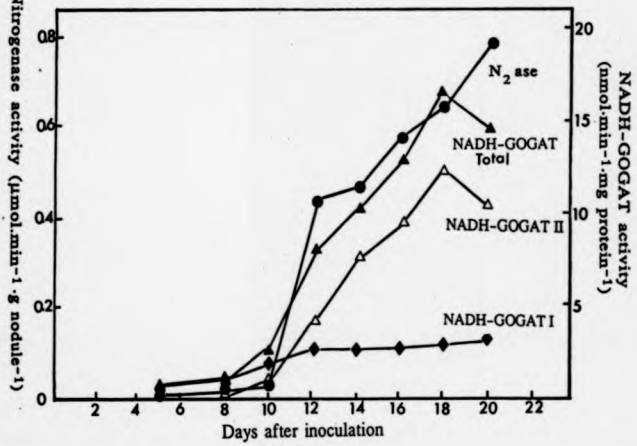


Fig.6.1. Changes in the activities of nitrogenase and the two isoenzymes of NADH-GOGAT during nodule development. The total NADH-GOGAT specific activities and the relative proportions of the two isoenzymes determined in Fig.6.2., were used to calculate the separate specific activities of NADH-GOGAT I and NADH-GOGAT II at each point.

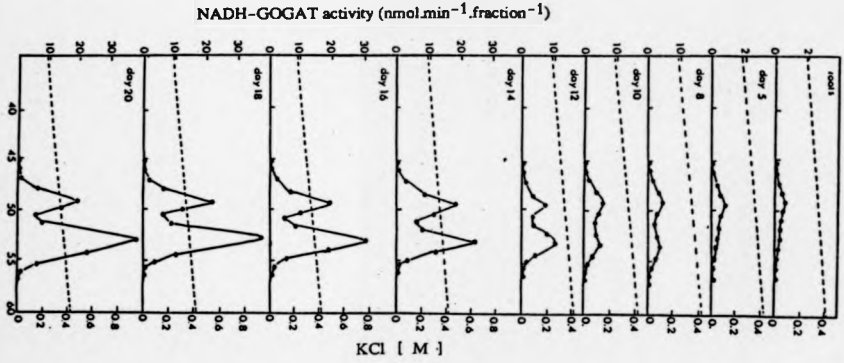


Fig.6.2. Changes in the relative activities of NADH-GOGAT I and NADH-GOGAT II during nodule development determined by activity elution profiles from an HPLC ion-exchange column

2. Changes in amount of nodule specific proteins and nitrogenase during nodule development.

Proteins extracted from root nodules at different stages of development were run by SDS-polyacrylamide gel electrophoresis (Fig.6.3.) The result revealed that the two most abundant nodule-specific proteins, leghaemoglobin (M_r of 14,000) and uricase (M_r of 35,000), both initially appeared at day 10 and then increased in abundance up to day 20. Protein bands with M_r of 200,000 (perhaps corresponding to NADH-GOGAT) were observed starting from day 12 and also increased in abundance at the same time course as uricase and leghaemoglobin. Some other proteins from the SDS-PAGE were also found to increase in abundance during nodulation, whilst most proteins were observed to be constant in abundance and only a few were found to decline during development.

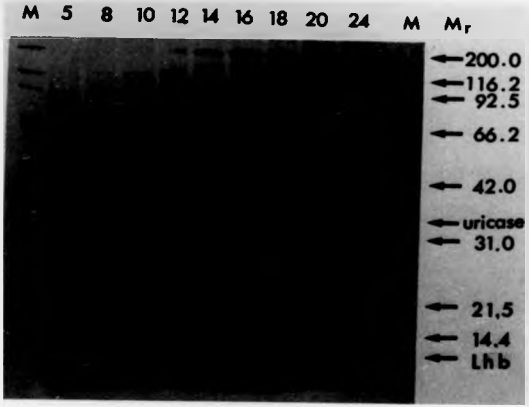


Fig.6.3. SDS-polyacrylamide gel of crude extracts of nodules at different stages of development. 10% SDS-polyacrylamide gel was run and stained with Coomassie blue. The number on the top of each track stands for the days after inoculation.

Nodule specific enzyme proteins induced at a similar time course to nitrogenase during nodule development were also observed by "Western" blot analysis. SDS-polyacrylamide gels of nodule extracts from different stages of development were blotted onto nitro-cellulose filters followed by incubating separately with nitrogenase antibody and a typical ammonia assimilatory enzyme uricase antibody, and anti-NADH-GOGAT antiserum obtained in this study. The filters were then incubated with [^{125}I]-protein A as described in Chapter 2. The exposed X-film from nitrocellulose filter (Fig.6.4. Fig.6.5. and Fig.6.6.) showed that the induction of nitrogenase and uricase occurred at the same time course during nodule development. They both appeared at day 10 and then increased in abundance up to day 20 and then decreased. There were hybridizing bands arround M_r of 200,000 using the anti-NADH-GOGAT antibody, which appeared from day 10 but the intensities of bands did not seem to be increased gradually, and another protein with M_r of about 40,000 was observed from this Western blotting filter and appeared to be increased in abundance gradually during nodule development. The explanation could be either that the purified protein with M_r of 200,000 from SDS-polyacrylamide gel was contaminated with some protein with M_r of 40,000 and therefore the NADH-GOGAT antibodies are not monospecific and are recognizing this contaminant or that the antibodies are specific for NADH-GOGAT but that the enzyme is degraded to a M_r of 40,000 protein in these crude extracts.

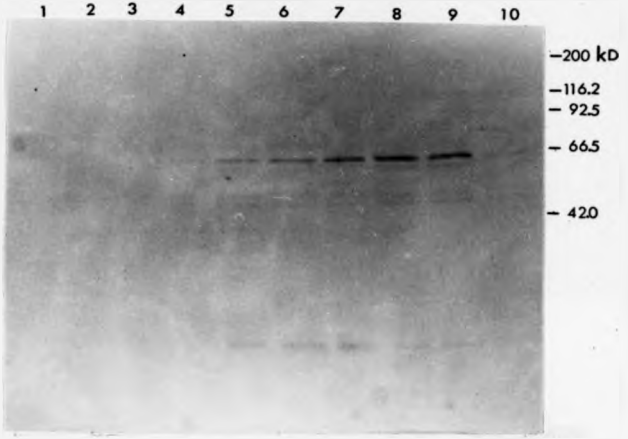


Fig.6.4. Western blotting of nitrogenase from crude extract of root nodules during nodule developmen. 1. day5, 2. day8, 3.day10, 4. day12, 5. day14, 6. day16, 7.day18, 8.day20 10. day24 after inoculation. The antibody was raised to component II of nitrogenase from *R.leguminosarum* bv *leguminosarum* and was kindly supplied by Dr.T. Bisseling (Agriculture University, Wageningen, The Netherland).

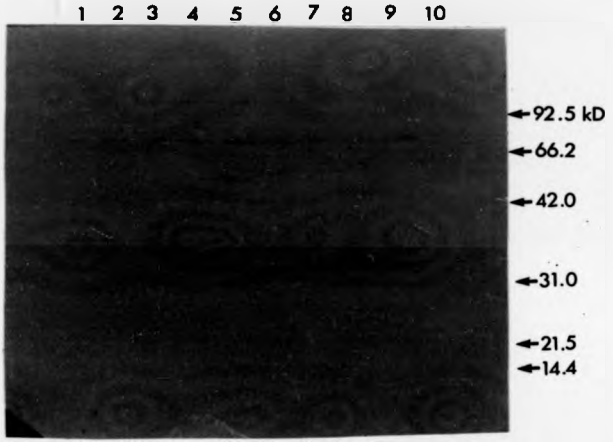


Fig.6.5. Western blotting of uricase from crude extract of root nodules during nodule development. 1. day5, 2. day8, 3.day10, 4. day12, 5. day14, 6. day16, 7.day18, 8.day20 10. day24 after inoculation. The antibody was raised to purified soybean nodule uricase and was kindly supplied by Prof. D.P.S.Verma (McGill University, Montreal, Canada).

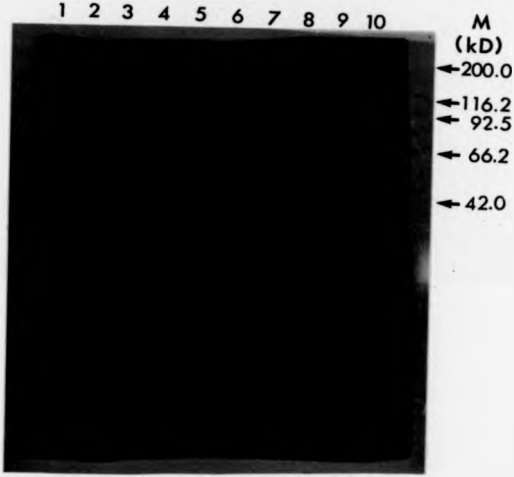


Fig.6.6. Western blotting of NADH-GOGAT from crude extract of root nodules during nodule development. 1. day5, 2. day8, 3. day10, 4. day12, 5. day14, 6. day16, 7. day18, 8. day20 10. day24 after inoculation.

3. A Comparison of Changes in NADH-GOGAT activity in nodules inoculated with *Rhizobia* Fix^- mutant CE108 and wild type CE3.

Five-day old *P.vulgaris* seedlings were inoculated with Fix^- mutant CE108 and wild type CE3 (Noel *et al.* 1984) (day 0). Plants were watered with sterilized -N nutrient solution every other day. 10mM NH_4^+ was given to two pots of CE108 inoculated plants on day 15 and 17. Nodules were harvested on day 12, 14, 16, 18 and 20 and assayed for nitrogenase and NADH-GOGAT activity. Both strains produced nodules of about the same size, but nodules formed with CE108 did not produce as much leghaemoglobin as formed with CE3, for the colour of nodules formed with CE108 were not very pink (Fig.6.7.). The leaves of CE108 inoculated plants were slightly yellow (Fig.6.8.). Nitrogenase activity in nodules formed with CE108 was observed to be less than 1% of that in nodules formed with CE3. (Fig.6.9a.). Nodule soluble protein levels in nodules formed with CE3 was higher than CE108 from early stages of nodule development and was about twice that in nodules formed with CE108 up to day20 (Fig.6.9b). Although activities of NADH-GOGAT isoenzymes were detectable in nodules formed with CE108 at the same time as CE3 and the level of activities of two isoenzymes was found to be the same as CE3 at early stages (day12), specific activities of both isoenzymes of NADH-GOGAT were not increased throughout nodule development, and

specific activity of NADH-GOGAT II was found to be decreased (Fig.6.10.). When 5mM $(NH_4)_2SO_4$ was given to plants inoculated with CE108 on day15 and 17, the specific activity of NADH-GOGAT I was almost constant but NADH-GOGAT II was slightly decreased (Fig.6.10a and b.). The proportion of two isoenzymes in nodules formed with two types of *Rhizobium* were determined by separating the enzymes by HPLC ion-exchange chromatography (Fig.6.11.).

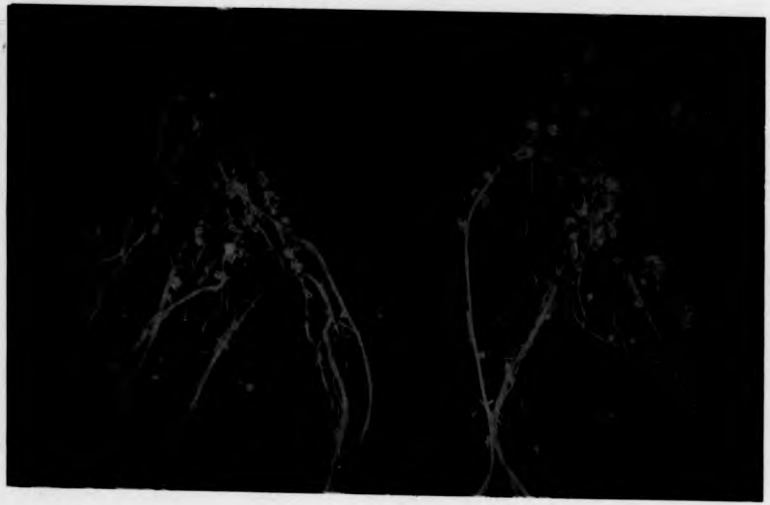


Fig.6.7. Comparison of root nodules formed with *Rhizobium* Fix$^-$ mutant CE108 (left) , and wild type CE3 (right) on day 20 after inoculation.

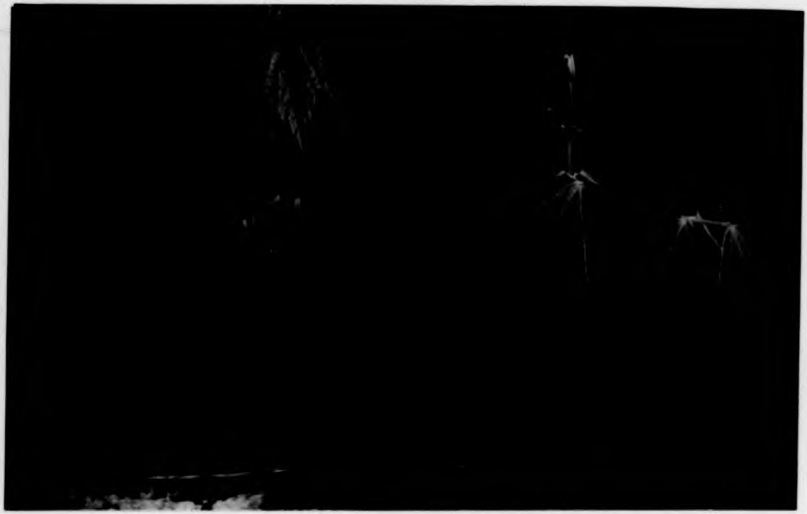


Fig.6.8. Comparison of plants inoculated with *Rhizobium* Fix$^-$ mutant CE108 (left) ,and wild type CE3 (right) on day 20 after inoculation.

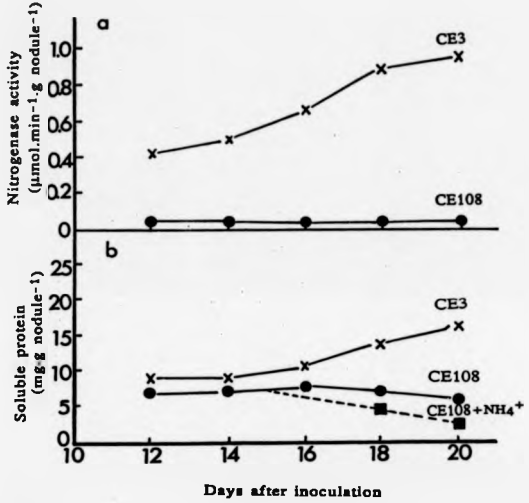


Fig.6.9. Comparisons of acetylene reduction and nodule soluble proteins in nodules formed with either *Rhizobium* Fix$^-$ mutant CE108 or wild type CE3, during nodule development. Assays began on day12, 14, 16, 18, and 20 after inoculation. 10mM NH_4^+ was given to nodules formed with CE108 on day15 and day 17.

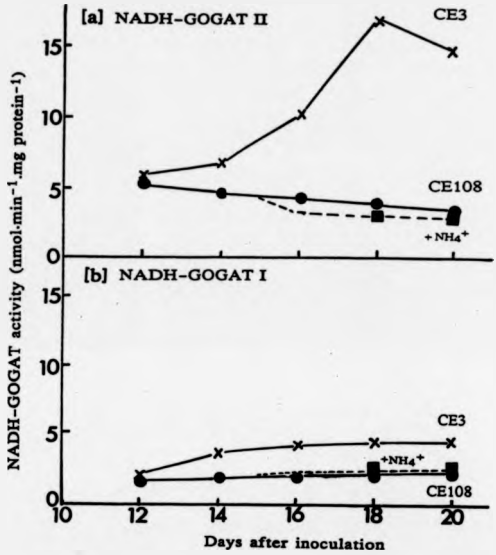
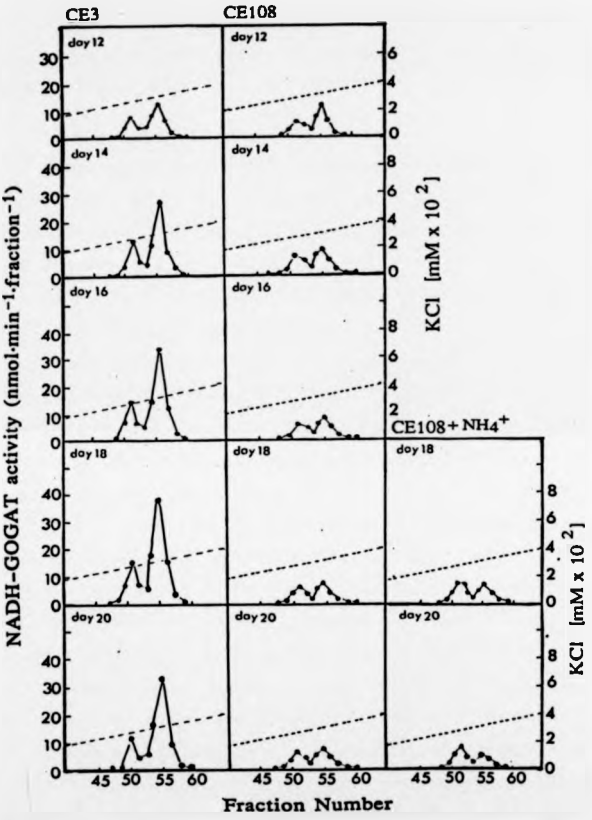


Fig.6.10. Comparisons of specific activity of two NADH-GOGAT isoenzymes from nodules formed with *Rhizobium* Fix$^-$ mutant CE108 and wild type CE3 during nodule development. Assays began on day12, 14, 16, 18, and 20 after inoculation. 10mM NH_4^+ was given to nodules formed with CE108 on day15 and day 17.

Fig.6.11. Changes in the relative activities of NADH-GOGAT I and NADH-GOGAT II in nodules formed with *Rhizobium* Fix$^-$ mutant CE108 and wild type CE3 during nodule development, determined by activity elution profiles from an HPLC ion-exchange column.


CE3
CE108
CE108+NH4+
day 12
day 14
day 16
day 18
day 20
NADH-GOGAT activity (nmol·min-1·fraction-1)
KCl [mM x 10-2]
Fraction Number
45 50 55 60

4. Changes in NADH-GOGAT activity in nodules formed under 80% Ar : 20% O_2:

Five-day old seedlings of *P. vulgaris* were inoculated with *R. leguminosarum bv phaseoli* (day 0). After giving 80% Ar : 20% O_2 to seven of the pots and 80% N_2 : 20% O_2 to the other five pots on day 5, all pots were sealed to keep the plants exposed to the desired atmosphere as shown in Fig.6.12. The atmospheres were checked by gas chromatography. First nodule samples from both conditions were harvested on day 15. On this day some were transferred to N_2: O_2 from Ar whilst some from N_2 were transferred to Ar and some from Ar were given 10mM NH_4^+ as described in Methods. Then nodules were harvested on day 16 and 19. All samples harvested were assayed for nitrogenase activity, soluble proteins and for NADH-GOGAT two isoenzymes activities (Fig.6.13. and Fig.6.14.). The elution profiles of the two isoenzymes of NADH-GOGAT from nodules grown under different conditions are shown in Fig.6.15. The nodules formed under Ar:O_2 were similar in appearance to nodules grown under N_2:O_2. From Fig.6.13. it can be seen that the activity of nitrogenase in nodules grown under Ar:O_2 was active and increased from day15 to day19 whilst the soluble proteins remained almost constant throughout nodulation. The specific activities of two NADH-GOGAT isoenzymes were about the same as normal nodules on day 15 but both kept constant from day 15 to day 19 (Fig.6.14). When the nodules were given 10 mM NH_4^+, the nitrogenase activity, nodule soluble proteins were markedly reduced

and the nodules looked brown and shrivelled in appearance; the specific activity of NADH-GOGAT I was not affected whilst NADH-GOGAT II was reduced. When Ar treated nodules were transferred back to N_2:O_2, nitrogenase activity and both NADH-GOGAT isoenzymes were increased again although remained still lower than normal nodules. The level of soluble proteins in plant cells of nodules was also increased soon after transfer to N_2:O_2. When nodules were transferred from N_2:O_2 conditions to Ar:O_2, the activities of nitrogenase was reduced to the level of that of nodules grown under Ar:O_2 on day 19, and the activities of both NADH-GOGAT isoenzymes declined, especially NADH-GOGAT II. Supplied NH_4^+ had nearly no effect on the induction of two isoenzymes of NADH-GOGAT under Ar:O_2 but slightly decreased the specific activity of NADH-GOGAT II.

Fig.6.12. The Ar:O_2 effect experiment. 80% N_2:20% O_2 and 80%Ar : 20%O2 gas streams were given to the inoculated *P.vulgaris* on day 5.

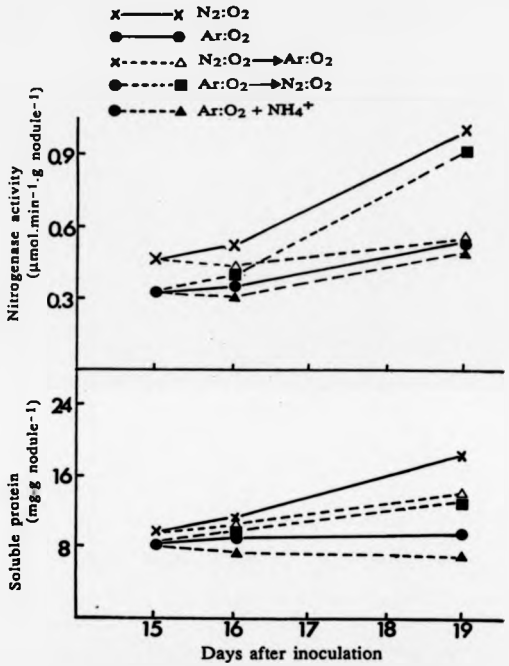


Fig.6.13. Effect of Ar:O_2 (80:20% v/v) treatment of the nodulated root systems of *P.vulgaris* on nitrogenase activity and the level of soluble proteins. N_2:O_2 or Ar:O_2 gas streams were maintained from day 5 to day 20 after inoculation.

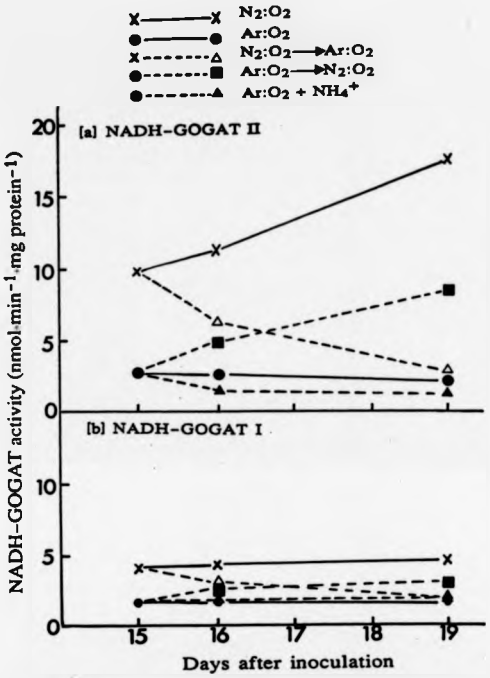


Fig.6.14. Effect of Ar:O_2 (80:20% v/v) treatment of the nodulated root systems of *P. vulgaris* on the specific activity of two NADH-GOGAT isoenzymes. N_2:O_2 or Ar:O_2 gas streams were maintained from day 5 to day 20 after inoculation.

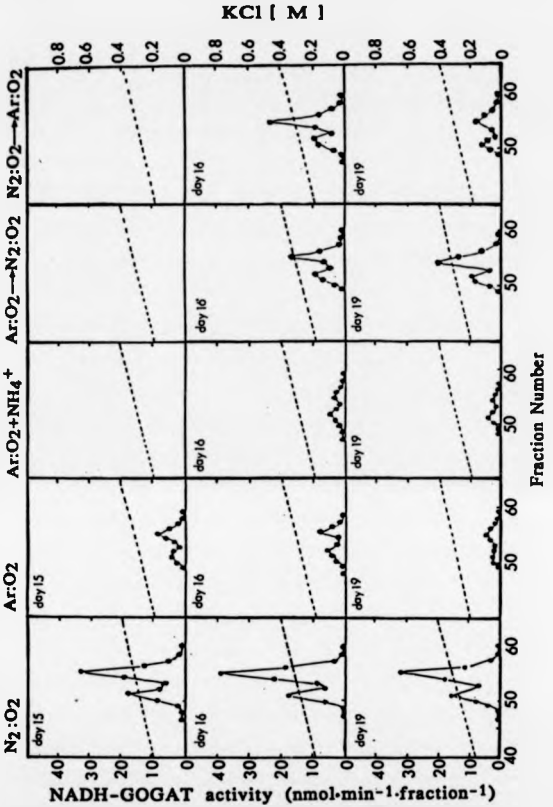


Fig.6.15. Changes in the relative activities of NADH-GOGAT I and NADH-GOGAT II in nodules formed under $Ar:O_2$ and $N_2:O_2$., determined by activity elution profiles from an HPLC ion-exchange column.

5. The effect of exogenously added NH_4^+ on activities of two NADH-GOGAT isoenzymes in uninoculated roots

The effect of NH_4^+ on activities of two NADH-GOGAT isoenzymes was determined by assaying the enzyme activity from roots of *Phaseolus vulgaris* grown under nitrogen starvation conditions. The beans were grown in vermiculite for about 5 days until the first leaf grew out, the cotyledons were picked off and the seedlings were transferred into two trays supported by wire mesh (hydroponic system) as shown in Fig. 2.3 The seedlings without cotyledons were grown in -N solution bubbled with fresh air for 2 days until the plants were nitrogen starved. On the third day, 10mM NH_4^+ was added to the solution in one tray for beans' growth for 24 h. The plants in the other tray were used as control. The roots from two trays were extracted separately in pH 7.6 Hepes buffer containing 0.5M sucrose, 1% β-mercaptoethanol, 10mM DTT, 1mM EDTA and 1mM PMSF. The extract was desalted by going through a 5ml Sephadex G-50 column. The desalted extract was chromatographed by HPLC ion-exchange column and the fractions were assayed for NADH-GOGAT activity. From the elution profile of the activities of NADH-GOGAT from the roots grown without adding NH_4^+(Fig 6.16a) and adding NH_4^+ (Fig 6.16b), it can be seen that only trace amount of NADH-GOGAT I was present in roots, and NADH-GOGAT II was not detected. After adding NH_4^+ to the plants, the specific activity of NADH-GOGAT I was increased less than 10%, NADH-GOGAT II was still not detectable.

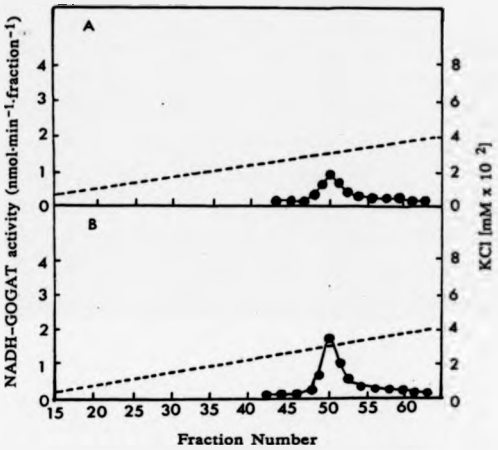


Fig.6.16. Ammonium effect on activities of NADH-GOGAT isoenzymes in uninoculated roots determined by activity elution profile from an HPLC ion-exchange column. A. NADH-GOGAT from uninoculated roots without adding NH_4^+, B.NADH-GOGAT from uninoculated roots adding 10mM NH_4^+.

6. Organ-specificity of the two NADH-GOGAT isoenzymes

Roots, leaves, etiolated shoots, stems, cotyledons, plumules and radicals of *Phaseolus vulgaris* were extracted in the same way as nodules. The crude extracts from these tissues were run on HPLC ion-exchange column separately (Fig.6.17.). The fractions were assayed for NADH-GOGAT activity. NADH-GOGAT activity was not detectable in leaves, stems, cotyledons and plumules. Although, NADH-GOGAT I was detectable from both roots and radicals, the level of the activity from these two organs was very low. The specific activity of NADH-GOGAT I from roots or radicals was only about 1/20 of that from root nodules.

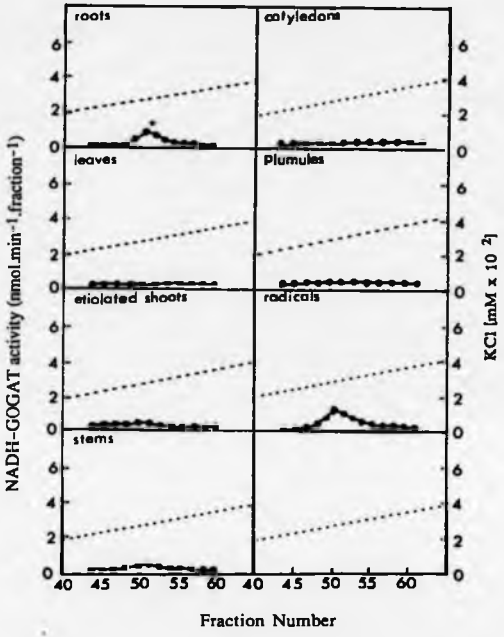


Fig.6.17. Elution profiles of two NADH-GOGAT isoenzymes from different organs of *P.vulgaris* from HPLC ion-exchange column.

6.3. Discussion

The activity of GS and NADH-GOGAT in the nodule cells has been shown to increase following the increase in the level of leghaemoglobin and in the activity of nitrogenase in the nodules (Robertson *et al.* 1975a and b, Werner *et al.* 1980). The results obtained in this Chapter confirmed the above observations and have shown that NADH-GOGAT I and NADH-GOGAT II are different in expression during nodule development of *P.vulgaris*. The activity of NADH-GOGAT I was increased at the early stage of nodulation but remained constant since day 14. Whilst the activity of NADH-GOGAT II was induced much more than NADH-GOGAT I and paralleled the induction of nitrogenase, leghaemoglobin and uricase during nodulation. The ratio of NADH-GOGAT II: NADH-GOGAT I was about 2:1 on the day 20 after nodulation. Therefore in *P.vulgaris* the increase in the activity of NADH-GOGAT during nodulation is mainly due to the induction of NADH-GOGAT II. NADH-GOGAT I was the only form when the inoculated root system on day 5 was assayed for the activities of two isoenzymes and was shown not only present in root nodules but also in roots and radicals; whilst NADH-GOGAT II was nearly undetectable from these organs. From the results obtained by HPLC ion-exchange chromatography of extracts from different organs of *P.vulgaris* (Fig.6.17)and the results from nodule developmental experiment (Fig.6.2), it appeared that NADH-GOGAT II

was the isoenzyme induced during nodulation and might be responsible for the assimilation of ammonium produced during dinitrogen fixation.

Nodules formed with Fix$^-$ mutant CE108 have the same size as effective nodules, but the colour of the nodules were not pink but yellowish brown and the leaves of CE108 inoculated plants were not as green as control plants (Fig.6.12. and Fig.6.13.). These observations suggest that there was lower level of leghaemoglobin in the nodules and there was not enough nutrient transported to the leaves for full chlorophyll synthesis when *P.vulgaris* was inoculated with Fix$^-$ mutant *Rhizobium*. The level of soluble proteins and two isoenzymes of NADH-GOGAT was also found to be lower than that in effective nodules. Moreover, nodules formed with CE108 seemed to have higher polysaccharide content since the extract of these nodules was very sticky and difficult to run through the Sephadex column. At the early stages of nodule development, the amount of soluble proteins and NADH-GOGAT two isoenzyme activities were found to be about the same as normal nodules, but the soluble proteins were slightly increased and activities of the two isoenzymes remained constant throughout nodule development. When NH_4^+ was given to CE108 formed nodules, the specific activity of NADH-GOGAT II was slightly decreased and the nodules started senescence soon after they were given ammonium. The results have shown that both NADH-GOGAT isoenzymes were synthesized despite the lack of nitrogenase activity in the bacteroids. However, the level of two isoenzymes was markedly reduced. A similar observation has been obtained when nodules were grown under $Ar:O_2$

conditions; the activities of two isoenzymes of NADH-GOGAT were expressed at the same time as normal nodules, but were not increased further during nodulation. When nodules formed under Ar:O_2 were transferred back to N_2:O_2 conditions, the two isoenzymes especially NADH-GOGAT II, were increased again. This was perhaps due to the recovery of nitrogen fixation when supplied with nitrogen. Therefore it could be proposed from the above two sets of experiments that nitrogen fixation might be a factor in regulating the level of two NADH-GOGAT isoenzymes. The results obtained in Ar:O2 treatment of nodulated root system were similar to that in the work on Ar:O_2 treated cowpea and lupin root nodules (Atkins *et al.* 1984). They found that Ar:O_2 treatment on attached nodules did not affect nodule growth, levels of plant cell and bacteroid protein, leghaemoglobin content, or nitrogenase activity but severely reduced activities of glutamine-utilizing enzymes, glutamate synthase, asparagine synthetase and de novo purine synthesis. The author proposed that ammonia production by *Rhizobium* bacteroids provided not only a source of nitrogen for growth but has a central regulatory role in maintaining the metabolic activity and functional integrity of the legume nodules.

The response of two NADH-GOGAT isoenzymes to applied ammonium in Ar treated nodules was also found to be similar to that in CE108 formed nodules; exogenous NH_4^+ supplied to plants could not induce activity of NADH-GOGAT but caused the nodule senescence. When

NH_4^+ was given to uninoculated roots, NADH-GOGAT II was still undetectable, the specific activity of NADH-GOGAT I was slightly increased. Therefore the exogenous ammonium has no effect on the induction of NADH-GOGAT II, the level of the expression of NADH-GOGAT II was found to be related with nitrogen fixation. It was clearly shown when Ar:O_2 treated nodules were transferred to N_2:O_2 conditions, the specific activities of two NADH-GOGATs, especially NADH-GOGAT II was dramatically increased and when nodules were switched off N_2 to Ar, the specific activity of NADH-GOGAT II was markedly reduced.

Chapter 7

Attemping the Molecular Cloning of Gene(s) Encoding NADH-GOGAT of *Phaseolus vulgaris* L.

7.1.Introduction

Studies at the molecular level have shown that GS in higher plants is encoded by a small nuclear multigene family (Cullimore *et al.* 1984, Tingey *et al.* 1987). In *P.vulgaris* GS is encoded by four expressed genes (gln-α, gln-β, gln-γ and gln-δ) which encode three cytosolic GS subunits (α, β and γ) and a plastid located subunit (δ) (Cullimore *et al.* 1984, Gebhardt *et al.* 1986, Lightfoot *et al.* 1988, Lara *et al.* 1984, Bennett and Cullimore unpubl.). In nodules the appearance of the nodule specific GS isoenzyme during nodule development (Cullimore et al, 1984) has been shown to be due to the expression of a specific gene. This gene, gln-γ is expressed only in nodules whereas the other three genes are expressed additionally in other organs(Cullimore *et al.* 1984, Gebhardt *et al.* 1986, Lara *et al.* 1984). The subunits then assemble into a number of separable isoenzymes in their respective compartments (Bennett and Cullimore, unpubl.). In contrast to the work on GS very little is known of the coding and gene expression of GOGAT in higher plants; to date no clones have been obtained for either the ferredoxin-dependent or NADH-dependent GOGATs.

However, the clones encoding NADPH-GOGAT gene from *E.coli* K-12 have been obtained and sequenced (Oliver *et al.* 1987). NADPH-GOGAT from *E.coli* is an aggregate of four catalytically active dimers. Each dimer consists of two nonidentical subunits whose estimated M_rs are 135,000 and 53,000 (Miller and Stadtman, 1972). The recombinant

plasmid pRSP20 carries the *E.coli* structural genes coding for both large and small subunits which are tightly linked (Oliver *et al.* 1987). Despite the differences in molecular weight and number of polypeptides between *E.coli* NADPH-GOGAT and *P.vulgaris* NADH-GOGAT, perhaps there is some conserved regions as there is some evidence which suggests that glutamine amido transferases have some conserved regions (Wootton, pers. commun.). GOGAT is a member of glutamine amido transferases. Therefore NADPH-GOGAT gene from *E.coli* could be used as a probe to screen for clones encoding NADH-GOGAT gene from higher plants.

We propose to obtain cDNA clones encoding NADH-GOGAT of *P.vulgaris* and to use these clones to study the number of genes that encode this enzyme and their expression in root nodules, particularly in relation to the nitrogen supply and the expression of the GS genes.

The strategy for cloning the genes involves preparing a cDNA library to high molecular weight polyA^+ RNA obtained from root nodules and screening the clones using the probes prepared from *E.coli* GOGAT clones. Potential *P.vulgaris* GOGAT clones would then be identified by hybrid select translation and immunoprecipitation of the protein products. GOGAT clones would be sequenced and used to study the structure of the gene(s) in *P.vulgaris* and the expression of the enzyme.

7.2. Results

1. Preparation of polyA$^+$ RNA and higher molecular weight mRNA

1) Separation of PolyA$^+$ RNA from total RNA

Total RNA was extracted as described in Methods. PolyA$^+$ RNA was purified by oligo-dT cellulose chromatography. Fig 7.1 shows the result of the separation of polyA$^+$ RNA from the total RNA by oligo-dT cellulose chromatography. The absorbances at 260nm of the fractions were checked by a UV monitor. From the profile it can be seen that a major peak of ribosome RNA was washed off directly from the column; PolyA$^+$ RNA was absorbed onto the oligo-dT on the column and a small peak of polyA$^+$ RNA was then eluted from the column with oligo-dT elution buffer.

2) Size-fractionation of PolyA$^+$ RNA by sucrose gradient centrifugation

PolyA$^+$ RNA isolated from nodules of *Phaseolus vulgaris* L. was separated into different size classes by size-fractionation by sucrose density gradient centrifugation. The gradient was checked by measuring the refractive index of one of the gradients with refractometer The result showed that the gradient was linear. After centrifugation, the gradients were fractionated into microfuge tubes and the absorbance at 260nm was checked at the same time. Unfortunately the peaks of RNA in both RNA marker tube and sample tube were not separatable, suggesting that sucrose

gradients were not satisfactory (Fig.7.2.). Fractions 5-16 however were saved, and used for *in vitro* translation.

3) *In vitro* translation of polyA$^+$ RNA

PolyA$^+$ RNA isolated from nodules of *P. vulgaris* was translated *in vitro* and the products characterized by SDS-polyacrylamide gel. The products of the *in vitro* translation (Fig. 7.3.) ranged in molecular weight from less than 30,000 to greater than 100,000. M_r of NADH-GOGAT is about 200,000 which suggests that the size of GOGAT mRNA should be about 6Kb. Fraction 12 was chosen for synthesizing cDNA, which translated to give the highest molecular weight product.

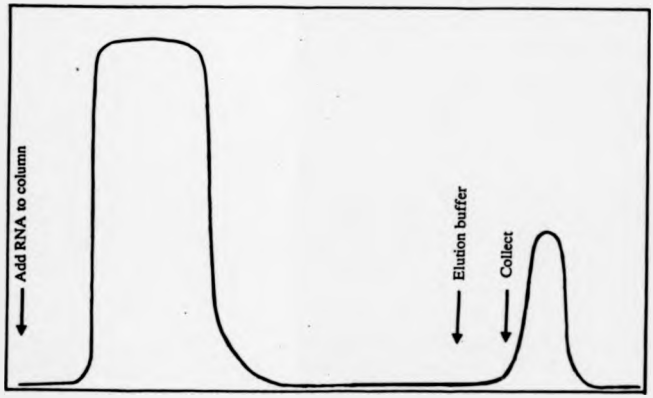


Fig.7.1. Elution profile of total RNA from root nodules of *P.vulgaris* from an oligo-dT column.

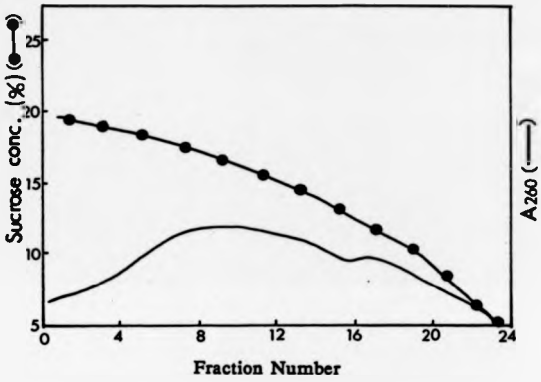


Fig.7.2. Size-fractionation of polyA$^+$ RNA by sucrose density gradient centrifugation

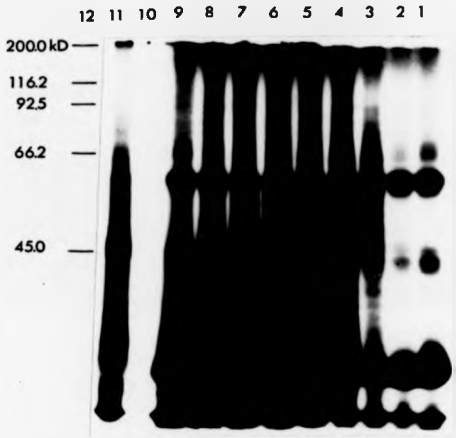


Fig.7.3. Autoradiography of SDS-PAGE of in vitro translation products of size-fractionated polyA+ RNA from root nodules of P.vulgaris. Track 1 to Track 9 are the translation products from size-fractionated polyA+ RNA No. 5-13, Track 10 :blank, Track 11: control yeast RNA, Track12: Molecular markers. Molecular weight markers are: ovalbumin (Mr 45,000),Bovine Serum albumin (Mr 66,2000), phosphorylase B (Mr 92,500), β-galactosidase (Mr 116,250) Myosin (Mr 200,000).

2. Preparation of cDNA library:

1) Synthesizing ds cDNA:

The M_r of cDNA was identified by alkaline agarose gel electrophoresis. The size of first strand and double strand cDNA synthesized from fraction 12 obtained by size fractionated polyA^+ RNA were only 1Kb or so, much smaller than expected. It suggested that the size of mRNA was not very large due to degradation during preparation. Therefore, mRNA fraction 19 which Dr. Cullimore obtained 3 years ago (Cullimore and Miflin, 1983) was used to synthesize cDNA. The size of synthesized cDNA from mRNA fraction 19 ranged from 0.6 to 4Kb (Fig.7.4.) It was still smaller than expected, the M_r of the majority was about 1.5Kb which suggested that mRNA obtained three years ago was also degraded during storage.

2) C-tailed double-strand cDNA:

A. trial tailing reaction from pUC8 cut by SmaI:

The different percentage of incorporation of dCTP into *Sma* I digested pUC8 was obtained at different reaction time as described in Methods and a plot of the incorporation of [α-^{32}P] dCTP against reaction time was made. (Fig 7.5). For 10 C-tails, 2% incorporation of dCTP was expected, the reaction should be carried out for 9min.

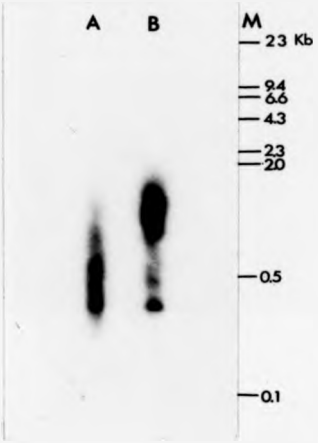


Fig.7.4. Alkaline agarose gel electrophoresis of synthesized first strand cDNA (Track A) and double strand cDNA (Track B). Molecular weight marker are Hind III digested λDNA.

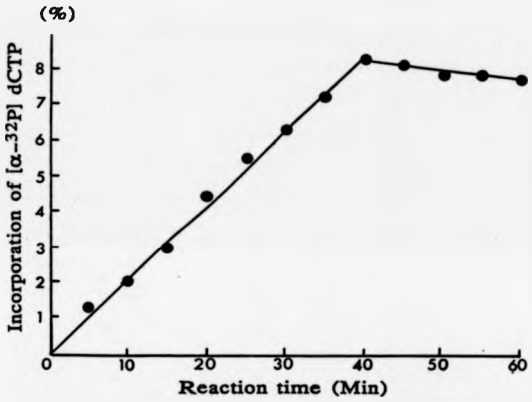


Fig.7.5. Trial tailing reaction for 10 C-tails from pUC8

B. C-tailing for ds-cDNA

The C-tailing reaction for ds-cDNA was carried out for 9 min as described in Methods. 10 C-tails were expected to be added to the ds-cDNA.

3). Transformation into both JM109 and JM83:

When cDNA obtained from mRNA fraction 12 was used, there were only a few colonies obtained on the transformation. However, when cDNA from mRNA fraction 19 which Dr. Cullimore obtained was used, in JM83 more than 100 white recombinant colonies in each plate appeared but in JM109 only a few colonies. JM83 and JM109 are both *Ecoli* K-12 derivatives. JM83 carries recA gene in the chromosome whilst JM109 does not. Therefore JM83 has much higher transformation efficiency. In all, 2000 clones were stored for the cDNA library.

3. Screening cDNA library:

1) Preparation of nick translation probe:

The restriction map of *E. coli* GOGAT gene (obtained as plasmid pGOG2 from Dr. Wootton) is shown in Fig.7.6. When cDNA was synthesized from mRNA, it starts from 3'end. The 3.6 Kb Hind III fragment of NADPH-GOGAT gene from *E. coli* was located at the 3' end and was used to make the probe since the size of double-strand cDNA was

only 1.5Kb, smaller than expected which should be 6Kb and therefore the cDNA library is unlikely to have any clones which contained full length of cDNA from root nodules. 3.6Kb fragment of NADPH-GOGAT gene was first isolated from an agarose gel of Hind III digested recombinant plasmid pGOG2 containing NADPH-GOGAT gene from *E. coli*. It was then ligated with pACYC184 and transformed into *E. coli* HB101 to amplify the 3.6 Kb fragment. The 3.6 Kb fragment was then isolated by elution from an agarose gel of Hind III digested pACYC184 recombinant plasmid (Fig.7.7). pACYC184 is totally different from vector pUC9 in cDNA library as there is no homology between the two vectors, even if there were remains of pACYC184 mixed with the 3.6Kb fragment, the probe would not hybridize to the vector of the cDNA library. Therefore pACYC184 was chosen to insert 3.6Kb fragment.

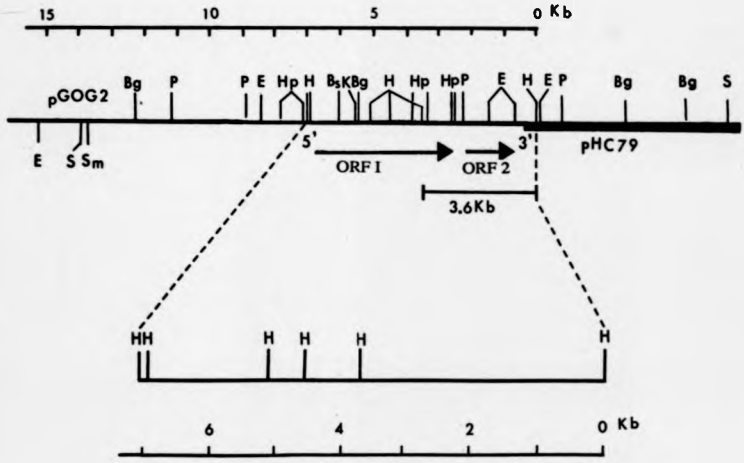


Fig7.6. Restriction map of *E.coli* NADPH-GOGAT gene showing the positions of the two open reading frames (ORFs) proposed to encode the large and small NADPH -GOGAT subunits. Restriction enzyme sites are: Bg (*Bgl* II), Bs (*Bst* EII),E(*Eco*R I) H (*Hind* III), Hp (*Hpa* I), K (*Kpn* I), P (*Pst* I), S (*Sst* I) Sm (*Sma* I)

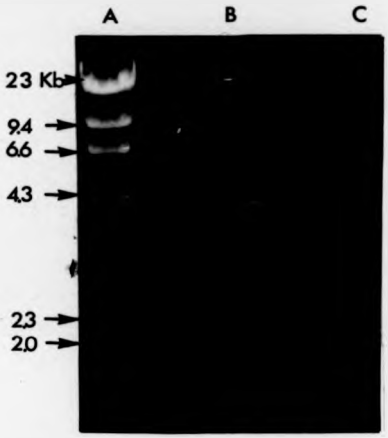


Fig.7.7. Isolation of the 3.6 Kb GOGAT fragment by elution from the gel of Hind III digested recombinant pACYC184 plasmid. Track A: Molecular marker of Hind III cut λDNA. Track B: Hind III digested recombinant pACYC184. Track C: Eluted 3.6 Kb NADPH-GOGAT gene fragment.

2) Preparation of SP6 transcription probe:

In pGEM3 there is a promoter highly specific for SP6 and T7 polymerases which direct specific initiation of the transcription reaction. Labelled transcripts produced with SP6 or T7 polymerase as probe exhibit several advantages compared to a nick-translated probe. The transcripts are single-stranded RNA and renaturation could not occur . The probe from transcription reaction has higher levels of specific labelling than nick translated probe. Moreover, the hybridization of RNA with DNA was stronger than that of DNA with DNA. The hybridization of RNA probe thus could be carried out under more stringent conditions, normally, the hybridization solution contains formamide to increase stringency and prevent RNA degradation. In order to make a higher radioactive RNA probe, the 3.6 Kb fragment was subcloned into pGEM3. Fig.7.8. shows the restriction map of pGEM3 + 3.6 Kb of *E.coli* NADPH-GOGAT gene fragment recombinant. However, there were two orientations in which the 3.6Kb fragment could ligate with the vector. The orientation of the 3.6Kb fragment was identified by mini-preparation of plasmid and digesting the recombinant plasmid with Pst I and running a mini-gel. Fig 7.9 shows that in tracks 4, 5, 6, 7, 9, 10, 11, a 4.3 Kb and a 2.4 Kb fragments were obtained, which suggests one orientation; in tracks 1, 2, 3, a 5.1 Kb and a 1.4 Kb fragment were obtained suggesting the other orientation. After SP6 transcription, it was shown that recombinant plasmid from first group produced full length RNA (Fig.7.10) but in the other direction, only small transcripts were obtained suggesting there could be a transcriptional stop in

this orientation. Recombinant plasmid from track 9 clone was used for making SP6 transcription probe.

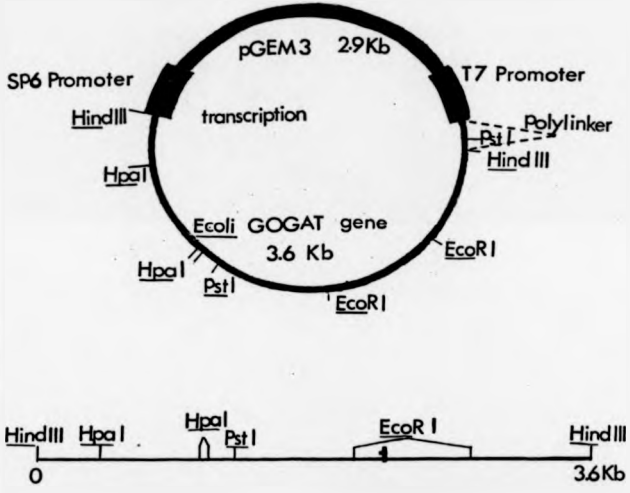


Fig.7.8. Restriction map of pGEM3 + 3.6Kb fragment of *E.coli* NADPH-GOGAT gene

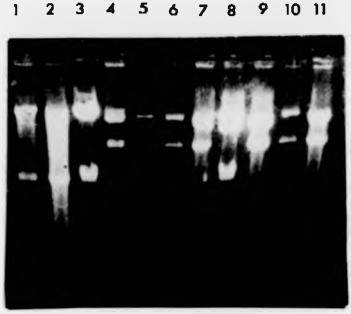


Fig.7.9. pGEM3 + 3.6Kb GOGAT fragment recombinant vector digested by *Pst* I Track1,2,3, and 8 give 1.4 + 5.3Kb fragment, Track 4, 5, 6, 7, 9, 10, and 11 produce 2.4 + 4.3 Kb fragment.

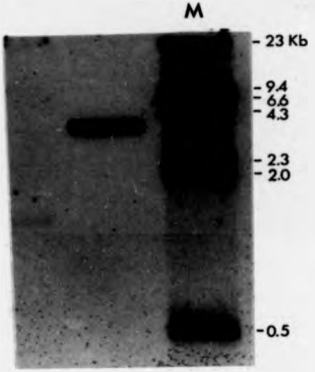


Fig.7.10. SP6 transcription of 3.6Kb fragment of *E.coli* NADPH-GOGAT gene. The marker used is ^{32}P labelled *Hin*dIII digested λDNA.

3. Hybridization of cDNA library with the probes:

There is expected to be only a low identity between NADPH-GOGAT gene from *E. coli* and NADH-GOGAT gene from higher plant so a low stringency was used for the hybridization. Fig 7.11 shows that one clone from one filter which contains 500 clones was found to hybridize strongly to the SP6 transcription probe after washing even at higher stringency (0.1xSSC, 0.1%SDS at 60°C) Fig.7.12. shows another clone from one filter which contains 500 clones was found to hybridize strongly to the nick translated probe after washing at high stringency (0.1xSSC, 0.1% SDS at 60°C).

10 clones altogether were chosen as putative GOGAT clones according to the results of hybridization of cDNA library with two kinds of probes. The 10 putative clones were double digested with *Hind* III and *Eco*R1 and the inserts were released from the vectors (Fig.7.13). Identification was carried out by blotting the gel onto a nitrocellulose filter and then hybridizing with SP6 transcription probe. It seemed that there were inserts hybridizing to the probe after washing at low stringency (Fig.7.14). All the hybridizing bands disappeared after the filter was washed at high stringency (Fig.7.15.).

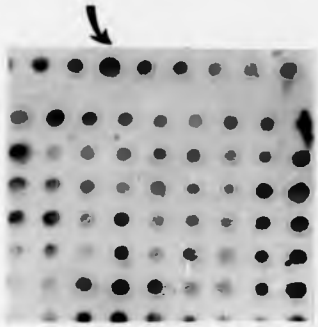


Fig.7.11. Colony hybridization of 500 recombinant nodule NADH-GOGAT cDNA clones to α-^{32}P-SP6 transcription probe from *E.coli* 3.6 Kb GOGAT gene fragment.

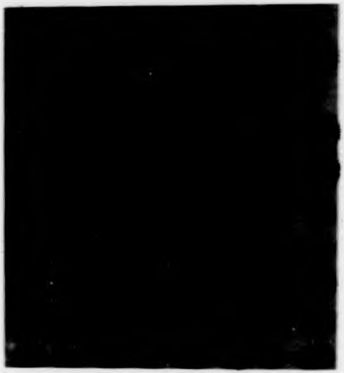


Fig.7.12. Colony hybridization of 500 recombinant nodule NADH-GOGAT cDNA clones to nick translation probe from *E.coli* 3.6 Kb GOGAT gene fragment.

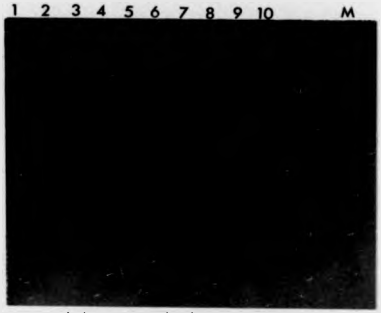


Fig7.13. Agarose gel electrophoresis of mini plasmid preps of potential 10 NADH -GOGAT cDNA clones (1-10) and marker clone pGEM3 + 3.6Kb fragment of *E.coli* NADH-GOGAT genes(M), digested by *Hind* III and *Eco*R1.

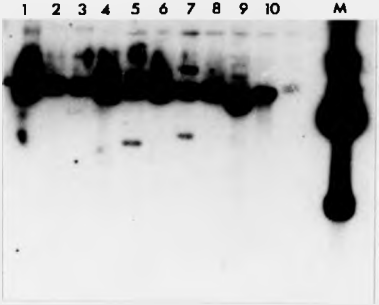


Fig7.14. Southern blot of 10 mini plasmid preps of potential NADH-GOGAT cDNA clones (1-10) and marker clone (M), digested by *Hind* III, *Eco*R1 and hybridizedwith the SP6 transcription probe and washed at low stringency (6xSSC, 0.1% SDS at 50°C).

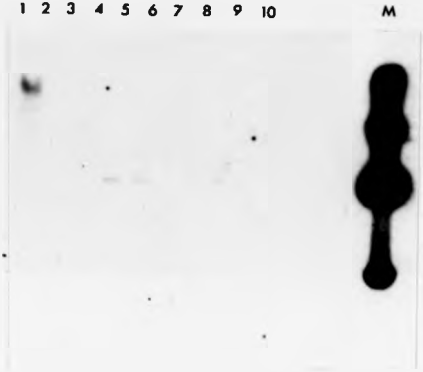


Fig7.15. Filter as shown in Fig7.14. but after washing at high stringency (0.1xSSC, 0.1%SDS at 60°C)

7.3. Discussion

A cDNA library has been constructed from double-stranded cDNA synthesized by the method of Gubler and Hoffman (1983), from size-fractionated polyA$^+$ RNA obtained from sucrose gradients. The library has been screened with the probes either nick translated labelled *E. coli* GOGAT gene fragment or labelled RNA transcripts from SP6 transcription of *E. coli* GOGAT gene fragment. However, the result was not satisfactory it looked as if none of the inserts hybridized to the *E. coli* GOGAT probes. The reason perhaps was any of the following:

A. Most inserts in cDNA clones were too small due to mRNA being degraded.

B. SP6 transcription probe was not full length, either degradation ocurred or transcription was stopped at several terminators.

C. Probe made from *E.coli* GOGAT gene does not recognize the plant GOGAT and the colony hybridization result was spurious.

To obtain cDNA clones encoding NADH-GOGAT gene of root nodules of *P.vulgaris* was the original aim of my Ph.D project. Because of the large size of the enzyme, low abundance in soluble proteins of the root nodules, instability of the enzyme and no suitable probe available, no clones encoding NADH-GOGAT gene was obtained. However NADH-GOGAT clones could be obtained from the following different strategies:

a. Construct a cDNA library in expression vector Lambda vector gt11. The expression library could then be screened with NADH-

GOGAT antibody.

b. Sequence NADH-GOGAT protein and synthesize an oligonucleotide to screen cDNA library.

c. Hybrid select translation of batches of 50-100 clones and identify the products with NADH-GOGAT antibody.

Chapter 8

Concluding Discussion

Concluding Discussion

Evidence has shown that NADH-GOGAT and Fd-GOGAT are two distinct enzyme proteins. From *A. thaliana* mutants and barley mutants, ferredoxin- and NAD(P)H-dependent glutamate synthase appear to be coded for by different genes (Suzuki and Gadal 1984). The relationship between the two forms of GOGAT in root nodules in relation to ammonium assimilation is not clear. NADH-GOGAT in root nodules has been reported to be induced in parallel with the activity of nitrogen during nodulation (Robertson *et al.* 1975), suggesting an overall role of this enzyme in nitrogen fixation. Moreover, in our experiments NADH-GOGAT from both *P.vulgaris* and soybean root nodules were found to be more active than Fd-GOGAT. This suggests that NADH-GOGAT probably plays a more important role than Fd-GOGAT in ammonium assimilation in root nodules.

We have discovered that NADH-GOGAT occurs as two distinct isoenzymes in the plant fraction of root nodules of *P.vulgaris*. Evidence for this statement are 1). two NADH-GOGAT activity peaks can reproducibly be separated from crude nodule extracts by ion-exchange chromatography and as each peak reruns in its original position on the column (Fig 3.2.). It appears that the two activities are separate entities and are not interconvertible in cell-free extracts, 2). the kinetic properties of the two activities show slight differences (Table 4.3.). 3)

the two activities are regulated differently during nodule development (Fig 6.2.) and 4) the cellular locations of the two NADH-GOGAT isoenzymes in nodules are different (Fig.5.4.and Fig.5.5.). It is extremely unlikely that either of the two NADH-GOGAT isoenzymes arise from the bacteroid fraction of the nodule because of the following reasons: 1) Care was taken not to extract the bacteroids, which pellet in the centrifugation of the crude nodule extracts (Awonaike *et al.* 1981, Suzuki *et al.*, 1984), and a marker enzyme of bacteroids, β-hydroxybutyrate reductase could not be detected in the extracts, indicating that there was no bacteroids contamination. 2) It has previously been shown that most activity of NADH-GOGAT in *P. vulgaris* nodules occurs in the plant cell cytoplasm and very little activity is associated with the bacteroids (Awonaike *et al.* 1981). Recent experiments have demonstrated that the NADH-GOGAT is present only in host plant cells, and absent from bacteroids (Suzuki and Gadal 1984). 3) The structure and coenzyme specificities of the two isoenzymes are similar to other plant NADH-GOGATs and not to most bacterial GOGATs (Suzuki and Gadal 1984).

Determination of the molecular weights of the two isoenzymes by SDS-polyacrylamide gel electrophoresis and gel-filtration chromatography has shown that both isoenzymes have the same molecular weight of about 200,000 for their native form and denatured form. Therefore the two isoenzymes are both monomeric proteins. The molecular weights of these two isoenzymes are the same as the M_r of

NADH-GOGAT from alfalfa root nodules (Anderson *et al.* in press) and similar to the M_rs determined for NADH-GOGATs isolated from other higher plants and *Chlamydomonas* (Suzuki and Gadal 1984). A slight difference in the elution position of the two isoenzymes was observed from the HPLC gel filtration column (Fig.3.3.) but whether this difference in elution position reflects actual differences in their Mrs is uncertain; no difference could be detected in their positions on SDS-polyacrylamide gels (Fig.4.6.). Kinetic characteristics of the two NADH-GOGAT isoenzymes from *P.vulgaris* fall within the range of values obtained for the kinetic properties of other plant NADH-GOGATs. However, a comparison of the kinetic characteristics of the two *P.vulgaris* NADH-GOGATs have revealed several differences, notably in their K_ms for L-glutamine, 2-oxoglutarate and NADH and in their pH optima (Table 4.2). The differences in kinetic properties of the two NADH-GOGAT activities confirmed that they were two isoenzymes and they might play different physiological roles in the root nodules.

The activities of both isoenzymes were found to be strongly dependent on NADH as reductant; only a very low activity could be supported by NADPH (Table 4.3.) and neither isoenzyme showed any activity with ferredoxin. The reductant specificities of these two isoenzymes are therefore similar to the specificities of other pyridine nucleotide dependent GOGATs that have been purified from higher plants and *Chlamydomonas* (Marquez *et al.* 1984, Matoh *et al.* 1979,

1980, Robertson *et al.* 1975). Studies on the enzyme isolated from etiolated pea shoots (Matoh and Takahashi 1980) and *Chlamydomonas* (Cullimore and Sims 1981, Marquez *et al.* 1984) have shown that neither FAD, FMN or ferredoxin could support activity, although some activity was detectable with reduced methyl viologen. We have not yet determined whether this artificial electron donor can substitute for NADH in this assay. There have been many reports of a GOGAT activity in higher plants which is non-specific for NADH and NADPH (Suzuki and Gadal 1984). Whether such a third type of higher plant GOGAT actually exists is still not clear, as an enzyme with this characteristic has not yet been purified.

It is interesting to notice that several cell fractionation studies, including work on cowpea nodules (Shelp *et al.*. 1983), have indicated that NADH-GOGAT activity may be present in the cytosol in addition to the plastids and Murillo *et al.* (1985) have speculated on the possibility that these activities may belong to separate isoenzymes. From our experiments we have shown that NADH-GOGAT is present as two isoenzymes, however, these two isoenzymes were both found to be located in plastids but perhaps in different cell types. NADH-GOGAT II is probably present in plastids of inner central infected cells and NADH-GOGAT I might be present mainly in plastids in the outercortex cells. The following observations could support these suggestions: 1) Activities of the two isoenzymes of NADH-GOGAT were both obtained in the plastid fraction of inner central cells and NADH-GOGAT II was

the major form (the ratio for NADH-GOGAT II and NADH-GOGAT I was 6:1) 2) the recoveries of two NADH-GOGAT isoenzymes in this experiment were found to be different, NADH-GOGAT I was recovered about 42% which was similar to the recoveries of two plastid marker enzymes, plastid GS and phosphoglycerate dehydrogenase, suggesting that these three enzymes are possibly located in the same type of cells whilst about 75% of NADH-GOGAT II was recovered. NADH-GOGAT II was a major form compared to NADH-GOGAT I and appeared to be nodule specific enzyme. Shelp *et al.* (1983) and Hanks *et al.* (1983) reported that the majority of the activity of enzymes associated with NH_4^+ assimilation was in the plastids of infected cells. Based on the result obtained by Reynolds *et al.* (1988) that AAT-P_2, a nodule specific isoenzyme for ammonia assimilation was located in plastids of infected cells, it could be speculated that NADH-GOGAT II is probably also located in infected cells. 3) About a third of NADH-GOGAT activity was found to be in outercortex of root nodules compared to total activity from root nodules and NADH-GOGAT I was found to be the major form (the ratio for NADH-GOGAT I and NADH-GOGAT II was 2:1).

NADH-GOGAT was obtained in the cytosol fraction and the percentage of NADH-GOGAT activity in the cytosol was observed to be about the same as that of plastid marker enzymes. This suggested that the activity of NADH-GOGAT in the cytosol was due to the breakage of plastids, yet we can not rule out the possibility that NADH-GOGAT

might be also present in the cytosol.

15% of activity of NADH-GOGAT in plastid fraction from the central tissue belonged to NADH-GOGAT I, which could not be explained by contamination of cells of outercortex because extreme care was taken when inner central cells were extracted from nodules. If a marker enzyme from outercortex cells would be assayed, the degree of contamination should be calculated. Thus we could speculate that NADH-GOGAT I might be also present in inner central cells of root nodules. Shelp *et al.* (1983) observed that activities of enzymes related to NH_4^+ assimilation were also present in the uninfected cell fraction, but these activities could be accounted for by contamination of 1% to 2% with infected cells, except for GOGAT, de novo purine synthesis enzymes, and uricase. This work suggested that NADH-GOGAT could be located in uninfected cells. Schubert (1986) proposed that low levels of GOGAT and purine biosynthetic enzymes are required for normal function of all cells and perhaps in *P. vulgaris* this activity is due to NADH-GOGAT I. The main role of GOGAT in nodules is undoubtedly to work in conjunction with GS to assimilate the newly-fixed dinitrogen, a role shown by ^{15}N-labelling data (Miflin and Lea 1980, Schubert 1986). GOGAT however also has minor roles in, for example, the assimilation of ammonia from phenyl propanoid metabolism and amino acid catabolism. The occurrence of two NADH-GOGAT isoenzymes might be of significance to accomplish all these functions.

It could be proposed that the two NADH-GOGAT isoenzymes had different roles in these processes resulting from different localization of two isoenzymes in root nodules. NADH-GOGAT II might be mainly responsible for assimilating ammonium from dinitrogen fixation, whilst the role of NADH-GOGAT I might be to assimilate ammonium produced from other cellular reactions.

However, GS, the first enzyme responsible for assimilation of ammonium produced during symbiotic nitrogen fixation in the nodule, was found almost entirely in the soluble fraction of the nodule (Awonaike *et al.* 1981, Boland *et al.* 1982). There is also evidence on GS localization using immunogold labelling that GS is located in the cytosol of infected cells (Verma *et al.* 1985). Because of the crucial role of GS in assimilating ammonium into organic form, it appears that a cytosolic location is reasonable and effective in preventing a toxic build up of ammonium. However, NADH-GOGAT II appears to be located mainly in the plastid of inner tissue together with most of other nodule specific ammonium assimilating enzymes in the two transport pathways (asparagine and ureides). Plastids might have a satisfactory environment and protecting system in maintaining activities of all those unstable enzymes such as NADH-GOGAT and the enzymes of purine synthesis. Therefore, it could be drawn the conclusion that plastids is an important organelle in ammonium assimilation in root nodules. These observations could suggest that there is a physical separation of the two enzymes of the glutamate synthase cycle through sub-cellular

compartmentation. Based on the observations on different locations of two isoenzymes of NADH-GOGAT in root nodules of *P.vulgaris* and the model presented by Schubert (1986) for the pathway and subcellular localization of amide synthesis and ureide biogenesis in nodules of ureide-producing legumes, a proposed pathway of ammonium metabolism in root nodules of *P.vulgaris* L. could be summarized as follows:

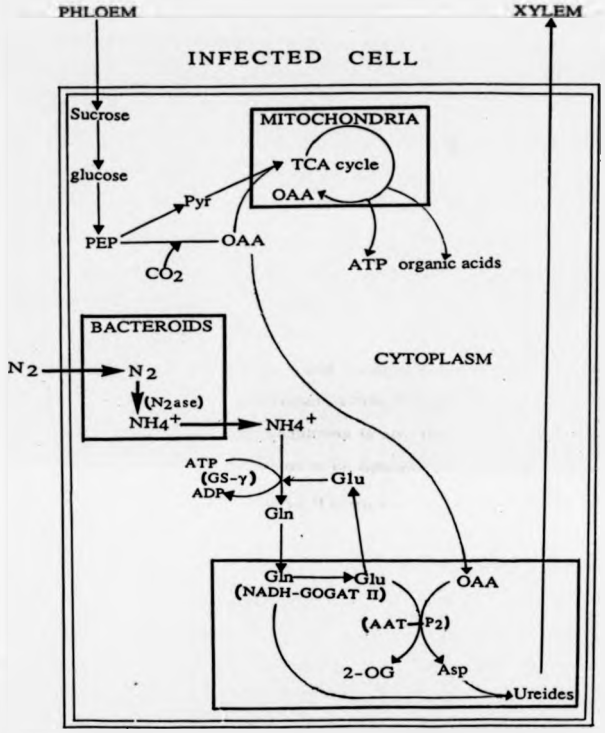


Fig 8.1. Proposed pathways of ammonium metabolism in root nodules of *P. vulgaris* L.

During nodulation of *P.vulgaris* the specific activity of NADH-GOGAT has been shown to increase about 27-fold and from Fig 6.3 it can be seen that this increase is due largely to the production of NADH-GOGAT II. Moreover in *P.vulgaris* the increase in activity of GS during nodulation occurs via the production of a nodule specific isoenzyme (Lara *et al.* 1983). Thus, both enzymes of the ammonia-assimilatory glutamate synthase cycle in *P.vulgaris*, appear to be regulated to increase their activities in root nodules by the increase in activity of specific isoenzymes. For GS, the appearance of this isoenzyme has been shown to be the result of the nodule-specific induction of one gene from the four-member GS multigene family in this species (Cullimore and Bennett, 1988). Whether the appearance of NADH-GOGAT II during nodulation is also the result of a nodule-specific gene induction remains to be determined. At present we are unsure whether NADH-GOGAT II is truly nodule-specific as the total NADH-GOGAT activity in roots and other organs appears to be very low and to be mainly composed of NADH-GOGAT I (Fig 6.17.).

The effect of $Ar:O_2$ treatment of nodulated root system of *P.vulgaris* was found to be similar to the work for lupin and cowpea (Atkins *et al.* 1984). They reported that the activities of enzymes involved in the ammonium assimilating pathway such as glutamine-utilizing enzymes, GOGAT, asparagine synthetase and de novo purine synthesis enzymes were all dramatically reduced when nodules were grown on $Ar:O_2$. They suggested that a far-reaching effect of the

production of ammonium by bacteroids on a wide range of enzymes might be possibly through control of protein turnover, rather than a high specific effect of ammonia. In our experiment, cultivation of nodulated *P. vulgaris* in Ar:O_2 and in the absence of any other exogenous N source accordingly resulted in the soluble proteins in nodule plant cells and NADH-GOGAT activity being reduced (Fig 6.13.). The specific activity of NADH-GOGAT II was reduced dramatically whilst the specific activity of NADH-GOGAT I was slightly reduced. The activity of two isoenzymes of NADH-GOGAT from nodules formed with Fix^- mutant of *Rhizobium* CE 108 was found to be reduced at the same degree as that in nodules formed with wild type *Rhizobium* under Ar:O_2 treated plants. In Ar: O2 grown nodules, nitrogen fixation was absent because of no dinitrogen supplied. In CE108 mutant formed nodules, nitrogen fixation was also absent but due to lacking nitrogenase. When exogenous NH_4^+ was given to the nodules, NADH-GOGAT activity was not increased but declined for both cases. All the observations suggest that nitrogen fixation itself but not the product ammonia could regulate the level of NADH-GOGAT activity. The supplied exogenous N from NH_4^+ did not overcome N deficiency but caused the senescence of root nodules. The effect of supplied NH_4^+ on Ar:O_2 treated nodulated root system was found to be slightly different from NO_3^- reported by Atkins *et al.*(1984) for cowpea nodules. They reported that addition of NO_3^- to Ar:O_2 plants initially caused protein increase in plant fractions of nodules but not bacteroid protein and this

was followed by senescence of the nodules. They explained that the extra N entering the nodule from the host following assimilation of NO_3^- was readily available for plant-cell protein synthesis but did not reach the bacteroids or was not readily utilized in their metabolism. Therefore senescence was not prevented despite the sufficiency of N in these nodules. In the case of NH_4^+ supplied, nodule soluble proteins were markedly reduced and senecence occurred soon after giving NH_4^+; it could be due to either the toxic effect of NH_4^+ to the plant cells or the induction of root nodule senescence by the supplied NH_4^+. Applied N, either NO_3^- or NH_4^+ could cause nodule senescence possibly due to nodule sensitivity to applied N as roots do not senescence by this treatment. The effect of NH_4^+ on nodules formed with CE108 was also found to be similar to nodules of Ar:O_2 grown plants. The observation was in agreement with the results of changes in activities of nodule enzymes of ammonium assimilation in an ineffective genotype of alfalfa [MnSa(In)] reported by Groat and Vance (1982). They reported that nodule proteins were reduced in response to applied N and the specific activity of GS and GOGAT either decreased or remained constant. The specific activity of NADH-GOGAT was found to be reduced especially NADH-GOGAT II in our experiments in correlation with the lack of nitrogen fixation in root nodules.

However, note that NADH-GOGAT was induced to some extent in both Ar:O_2 grown plants and Fix^- mutant formed nodules, although the level of both isoenzymes was decreased compared to normal fixing

nodules. This result obtained in our experiment was also in agreement with the results obtained by Lullien *et al.* (1987) and Sengupta-Gopalan and Pitas (1986). They observed that the induction of nodule specific GS and nodule-specific genes was independent of nitrogen fixing activity. Therefore, it could be proposed that some factors present in either *Rhizobium* or plant cells of the nodule, might be involved in regulating the initial expression of genes of nitrogenase, nodule-specific GS and NADH-GOGAT II.

From the studies on two isoenzymes of NADH-GOGAT, it can be seen that it is important to obtain cDNA clones coding for NADH-GOGAT of *P.vulgaris* to study the expression of two isoenzymes of NADH-GOGAT, to determine the number and structure of genes encoding NADH-GOGAT in *P.vulgaris* and to elucidate the molecular mechanisms regulating the enzymes in the glutamate synthase cycle. Because of the large size of the enzyme and problems with probes, we have not obtained clones encoding NADH-GOGAT. However as antiserum has been obtained and the biochemical properties of the enzyme has been studied, several strategies can be used to attempt to isolate cDNA clones encoding NADH-GOGAT genes in the future.

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